Title

Acylcarnitines as markers of exercise-associated fuel partitioning, xenometabolism, and potential signals to muscle afferent neurons

Journal

Experimental Physiology, 102(1)

ISSN

0958-0670

Authors

Zhang, Jie
Light, Alan R
Hoppel, Charles L
et al.

Publication Date

2017

DOI

10.1113/ep086019

Peer reviewed

Published in final edited form as:

Exp Physiol. 2017 January 01; 102(1): 48–69. doi:10.1113/EP086019.

Acylcarnitines as markers of exercise-associated fuel partitioning, xenometabolism, and potential signals to muscle afferent neurons

Jie Zhang[1], **Alan R. Light**[1], **Charles L. Hoppel**[2], **Caitlin Campbell**[3], **Carol J. Chandler**[3], **Dustin J. Burnett**[3], **Elaine C. Souza**[3], **Gretchen A. Casazza**[4], **Ronald W. Hughen**[1], **Nancy L. Keim**[3,5], **John W. Newman**[3,5], **Gary R. Hunter**[6,7], **Jose R. Fernandez**[6], **W. Timothy Garvey**[6], **Mary-Ellen Harper**[8], **Oliver Fiehn**[9,10], and **Sean H. Adams**[11,†]

[1]Anesthesiology Department, University of Utah School of Medicine, Salt Lake City, UT

[2]Pharmacology Department, Case Western Reserve University, Cleveland, OH

[3]USDA-ARS Western Human Nutrition Research Center, Davis, CA

[4]Sports Medicine Program, School of Medicine, University of California

[5]Department of Nutrition, University of California, Davis

[6]Department of Nutrition Sciences, University of Alabama, Birmingham, AL

[7]Human Studies Department, University of Alabama, Birmingham, AL

[8]Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON, Canada

[9]Genome Center –and- West Coast Metabolomics Center, University of California, Davis

[10]King Abdulaziz University, Biochemistry Department, Jeddah, Saudi-Arabia

[11]Arkansas Children's Nutrition Center –and– Department of Pediatrics, University of Arkansas for Medical Sciences

Abstract

With insulin-resistance or type 2 diabetes mellitus, mismatches between mitochondrial fatty acid fuel delivery and oxidative phosphorylation/tricarboxylic acid cycle activity may contribute to inordinate accumulation of short- or medium-chain acylcarnitine fatty acid derivatives (markers of incomplete long-chain fatty acid oxidation [FAO]). We reasoned that incomplete FAO in muscle would be ameliorated concurrent with improved insulin sensitivity and fitness following a ~14 wk training and weight loss intervention in obese, sedentary, insulin-resistant women. Contrary to this hypothesis, overnight-fasted and exercise-induced plasma C4-C14 acylcarnitines did not differ

[†]To whom correspondence should be addressed: Sean H. Adams, Arkansas Children's Nutrition Center, 15 Children's Way, Little Rock, AR 72202, shadams@uams.edu.

Competing Interests: The authors declare that they have no competing interests.

Authors' Contributions: Exercise study design (SHA, CLH, GAC, NLK, JWN, GRH, JRF, WTG, MEH, OF, CC, CJC, ECS); Neuron study design (SHA, JZ, ARL); Conducted research (SHA, JZ, ARL, CLH, CC, CJC, DJB, ECS, GAC, MBG, RWH); Analyzed data (SHA, SLH, JZ, ARL, CC); Wrote manuscript (SHA); all authors edited and provided input to the manuscript.

between pre-intervention and post-intervention phases. These metabolites all increased robustly with exercise (~45% of pre-intervention VO_{2peak}) and decreased during a 20 min cool-down. This supports the idea that, regardless of insulin sensitivity and fitness, intramitochondrial muscle β-oxidation and attendant incomplete FAO are closely tethered to absolute ATP turnover rate. Acute exercise also led to branched-chain amino acid (BCAA) acylcarnitine derivative patterns suggestive of rapid diminution of BCAA flux through mitochondrial branched-chain ketoacid dehydrogenase complex. We confirmed our prior novel observation that weight loss/fitness intervention alters plasma xenometabolites (i.e., cis-3,4-methylene-heptanoylcarnitine and γ-butyrobetaine [a co-metabolite possibly derived in part from gut bacteria]), suggesting that host metabolic health regulated gut microbe metabolism. Finally, we considered if acylcarnitine metabolites signal to muscle-innervating afferents: palmitoylcarnitine at concentrations as low as 1–10 μM activated a sub-set (~2.5–5%) of these neurons *ex vivo*. This supports the hypothesis that in addition to tracking exercise-associated shifts in fuel metabolism, muscle acylcarnitines act as exertion signals to short-loop somatosensory-motor circuits or to the brain.

Keywords

xenometabolome; xenobiotic; somatosensory nerve; T2DM; branched chain amino acids; muscle fatigue

Introduction

Human insulin resistance and type 2 diabetes mellitus (T2DM) are associated with less efficient/incomplete skeletal muscle long-chain fatty acid (LCFA) combustion, and are also characterized by impaired metabolic flexibility (defined as the shift away from LCFA oxidation toward carbohydrate metabolism in response to increased insulin)(see, e.g., (Mandarino *et al.*, 1996; Kelley *et al.*, 1999; Kelley, 2005; Ukropcova *et al.*, 2005)). These concepts are supported by cross-limb respiratory quotient (RQ) measurements (Kelley & Simoneau, 1994; Mandarino *et al.*, 1996; Kelley *et al.*, 1999), reduced exercise-associated whole-body plasma-derived LCFA oxidation in obese T2DM or insulin-resistant vs. obese controls (Blaak *et al.*, 2000; Mensink *et al.*, 2001), and a more limited combustion of isotopically-labeled LCFA and entry of the LCFA carbon into the TCA cycle in fasted T2DM subjects (Blaak & Wagenmakers, 2002). There is some evidence that uptake of LCFA is modestly reduced ~20–30% in insulin-resistant or T2DM muscle during fasting (Kelley & Simoneau, 1994; Blaak & Wagenmakers, 2002), but this observation is not universal (Kelley *et al.*, 1999) and in the postprandial state, muscle uptake does not differ (or is increased) relative to controls (i.e., (Kelley & Simoneau, 1994; Blaak & Wagenmakers, 2002; van Hees *et al.*, 2011; Moors *et al.*, 2012)). Thus, it is unlikely that reduced tissue uptake of LCFA can explain less complete LCFA muscle oxidation in the insulin-resistant or T2DM states.

The altered fatty acid oxidation in these states is reflected in part by a mismatch between mitochondrial fatty acid oxidation (FAO) in muscle and other tissues, relative to fuel availability (“mitochondrial overload”; (Koves *et al.*, 2008)): this leads to accumulation of lipid derivatives reflective of incomplete β-oxidation. This can manifest in higher

intramyocellular lipid in untrained individuals, which is highly correlated with insulin resistance (Jacob *et al.*, 1999; Krssak *et al.*, 1999; Malenfant *et al.*, 2001; McGarry, 2002; Mingrone *et al.*, 2003). Concentrations of plasma acylcarnitine derivatives and markers of incomplete LCFA β-oxidation are also increased in connection with insulin resistance, T2DM and poor blood sugar control (Genuth & Hoppel, 1981; Inokuchi *et al.*, 1995; Moder *et al.*, 2003; Adams *et al.*, 2009; Mihalik *et al.*, 2010). We proposed a potential role for acylcarnitines in triggering cell stress pathways associated with insulin resistance (Adams *et al.*, 2009), subsequently demonstrating pro-inflammatory actions of long-chain acylcarnitines in monocyte/macrophage cells (Rutkowsky *et al.*, 2014) and inhibitory actions on insulin signaling in cultured muscle cells (Aguer *et al.*, 2015). In addition to elevations in acylcarnitine accumulation, the lipotoxicity of insulin-resistance and T2DM can involve higher muscle content of diacylglycerol and ceramides—both implicated in inhibition of insulin signaling (reviewed in (Coen & Goodpaster, 2012)). Consistent with observations *in vivo*, incomplete LCFA β-oxidation is more evident in cultured myotubes isolated from obese, insulin-resistance individuals compared to lean controls (Aguer *et al.*, 2015), and in insulin-resistant rat myocytes (Koves *et al.*, 2008) highlighting the clear correlation between aberrant muscle LCFA metabolism and insulin resistance. Despite this, and the well-established role of muscle in whole-body insulin-stimulated glucose uptake, no biomarkers or metabolites that specifically reflect muscle LCFA β-oxidation dynamics have been identified. Such biomarkers would provide insight into the connection between fat metabolism and insulin sensitivity, and might be useful tools to assess diabetes risk, T2DM severity, or to gauge efficacy of interventions designed to ameliorate muscle insulin resistance. Furthermore, by characterizing muscle-abundant metabolites it may be possible to identify signaling molecules that are involved with local (i.e., neuronal) and systemic (i.e., tissue-to-tissue) cross-talk.

A major challenge to identifying selective biomarkers of muscle LCFA combustion is the paucity of comparative experimental models in which β-oxidation is altered exclusively or predominantly in muscle cells. One approach to identify candidate metabolites is to perform studies that challenge the muscle metabolic machinery to engage fat oxidation. Improvements in muscle mitochondrial function (i.e., more efficient LCFA combustion) and insulin sensitivity would be expected to change the patterns and concentrations of exercise-associated metabolites as reflected in the circulation. To this end, we employed a sub-maximal acute exercise paradigm to characterize exercise-induced plasma acylcarnitines in a human cohort of insulin-resistant, sedentary women before and after a weight loss and fitness intervention that markedly improved fitness and insulin sensitivity. To maximize our ability to identify changes in plasma metabolites specifically reflective of muscle metabolism, the acute exercise duration (30 min, sampling every 5 min.) and moderate intensity (~40–45% VO_{2peak}) used for metabolite profiling were designed to rapidly activate muscle work, lipolysis and ensure robust fatty acid oxidation (Romijn *et al.*, 1993; Brooks, 1997), while minimizing changes in liver β-oxidation (Fery & Balasse, 1983, 1986). It was hypothesized that acute exercise-induced plasma medium-chain fatty acylcarnitines (indices of incomplete LCFA β-oxidation) would be reduced concurrent with improved metabolic fitness and insulin sensitivity. The close link we observed between exercise and plasma acylcarnitine concentrations subsequently led to novel proof-of-principle studies testing

whether acylcarnitines could serve as signals to specific peripheral sensory neurons that interrogate the muscle beds. We speculate that metabolite communication plays an important role in providing the central nervous system (CNS) with information related to exertional fatigue, exercise duration and intensity.

Materials and Methods

Ethical Approval and Human Subjects Information

Extensive details regarding diet, recruitment, Test Week protocols, and other intervention-associated aspects for this cohort are provided in a recent report of oral glucose tolerance test metabolomics (Campbell *et al.*, 2014). A succinct summary is provided here.

All protocols were approved by the University of California at Davis Institutional Review Board, in alignment with the Declaration of Helsinki, and all subjects provided informed written consent. The study is listed in ClinicalTrials.gov (NCT01494025). To identify metabolites that are responsive to changes in insulin sensitivity due to improvements in fitness and body mass following a diet and exercise intervention, obese modestly hyperinsulinemic 30–50 year old females were recruited from the greater Davis and Sacramento, California communities. All participants were eumenorrheic, non-smoking, and sedentary (typical planned exercise <30 min. per week as self-reported). Body mass index (BMI) was between 30–37.5 kg/m^2 and participants were weight stable (<5% change in body mass over the previous 6 months). Insulin-resistance at the time of screening was identified through an overnight-fasted blood glucose and insulin, and an abbreviated oral glucose tolerance test (OGTT; fasting glucose plus a 2 hr glucose after consuming a standard 75 g glucose drink). Insulin resistance was defined as one or more of the following: (a) as per the American Diabetes Association guidelines for pre-diabetes, fasting glucose ≥100 and <126 mg/dL or 2-hour OGTT glucose ≥140 and <199 mg/dL; and/or (b) a target Quantitative Insulin Sensitivity Check Index (QUICKI) score <0.315, Homeostasis Model Assessment (HOMA) >3.67, or logHOMA >0.085. The latter criteria were derived using the upper limit of normal fasting glucose (100 mg/dL) and a serum insulin (15 μU/mL) that approximates the upper value of the third quartile of 72 normoglycemic men and women tested by the UC Davis Medical Center (UCDMC) Pathology Laboratory in the course of establishing their normal ranges for insulin. Exclusion criteria included any clinical signs of infection, chronic disease, personal history of cardiovascular disease, elevated blood pressure (>130/85 mm Hg), diabetes, regular medications other than oral contraceptives, and pregnancy or lactation. Sixteen participated through the first phase of the study; 1 subject was not compliant with the prescribed diet provided during either Test Week and was therefore excluded. Three subjects dropped following Test Week 1, prior to or during weight loss/exercise intervention, leaving 12 of 15 compliant subjects available for re-examination in Test Week 2.

Pre- and Post- Intervention Test Week Protocol

Participants completed testing before (“Test Week 1”) and after (“Test Week 2”) an exercise and weight loss intervention (14–17 weeks) designed to improve fitness and insulin

sensitivity (see (Campbell *et al.*, 2014); briefly described here). During each Test Week, subjects refrained from exercise and were weight-stable at the time of testing.

Test Week Diet—To minimize variability in metabolomics that could be influenced by differences in diet composition, during Test Week 1 and Test Week 2 the participants were provided lot-matched foods such that they ate identical diets for the Test Weeks (Campbell *et al.*, 2014). Diets were prescribed to maintain body mass during the specific Test Week using the Dietary Reference Intakes equation. Mass was determined daily during Test Weeks and small changes in prescription calories were made to maintain body mass within 5%. Study menus were prepared by a registered dietitian using the University of Minnesota's Nutrient Data System for Research (NDS-R) version 2009. Meals were prepared by the WHNRC Metabolic Kitchen and Human Feeding Laboratory using ProNutra software (Viocare Technologies, Inc.), guided by the 2005 Dietary Guidelines for Americans. Menus consisted primarily of shelf-stable (e.g., frozen, dried, canned) foods. During Test Weeks, participants were instructed to eat and drink only what was provided to them, and encouraged to eat study meals on site; however, for logistical reasons (e.g., work schedules) many meals were packed "to go." Self-reported adherence was determined with daily food diaries.

RMR (resting metabolic rate)—On Day 3 or 4 of each Test Week, participants reported to the WHNRC Physiology Laboratory for RMR measurements in the morning following a 12 hr overnight fast and having refrained from moderate to vigorous physical activity for 24 hr. They were made comfortable in a reclining chair and were instructed to sit quietly for 10 min. The subject was fitted with a facemask and RMR measured using indirect calorimetry (Parvo Medics TrueOne 2400 metabolic cart, Sandy, UT). Respiratory gas exchange measurements were made for the next 20–30 minutes and the average RMR and RQ was determined.

Body Composition Measurements—Weight was measured on an electronic scale to the nearest 0.1 kg with participants in light clothing, all jewelry removed, pockets emptied, and shoes removed. Height was measured using a wall mounted stadiometer. Body fat mass and fat-free mass were measured by dual energy X-ray absorptiometry (DXA, GE Lunar Prodigy Encore v10.5, Madison, WI).

Peak Exercise Test—On Day 4 or 5 of each Test Week, a graded cycle ergometer test (SRM ergometer, Colorado Springs, CO) was performed to determine peak oxygen consumption (VO_{2peak}). Participants arrived at the UC Davis Sports Medicine Clinic after consuming a standard breakfast (Menu 2 in Reference (Campbell *et al.*, 2014)) 2–3 hours prior to exercise. During Test Week 1, the participants received a resting ECG, a spirometry test, and a medical clearance exam by a UC Davis Sports Medicine Clinic physician, to ensure there were no health issues precluding exercise. For the exercise test, participants completed a 5 min warm up, followed by a graded exercise test to exhaustion (initial workload of 50W, increased by 20W every 2 min until volitional fatigue). VO_{2peak} was determined as the highest VO_2 (mL/kg/min) over a 30 s period. The VO_{2peak} measurement was replicated during Test Week 2.

Plasma Metabolite Profiling during Submaximal Exercise Test—Forty-eight hr after the peak exercise test, on Day 7 or 8 (depending on subject) of the Test Weeks, participants reported to the WHNRC Physiology Support Laboratory (PSL) in the morning following a 12-hr overnight fast and having refrained from any moderate to vigorous physical activity since the VO_{2peak} test. Subjects were fitted with a HR monitor (Polar Vantage NV™ model #1901001, Polar Electro Inc., Port Washington, NY). An intravenous catheter was placed in an arm vein (typically antecubital). The first blood sample was taken 5 mins after the catheter was placed and prior to exercise. After a brief 5 min warm-up involving pedaling without workload, subjects were fitted with headgear and nose clip for metabolic cart measurements. During Test Week 1, the tension on the cycle ergometer (Monark 828E, Sweden) was set to elicit an individualized workload (ca. 64W, average across all subjects) at 45% VO_{2peak} determined from the peak exercise test. Participants pedaled at the appropriate cadence of 50–60 rpm for 30 min. Between 0–5 min of exercise, indirect calorimetry values were checked to ensure the subject was working at an intensity of 45% VO_{2peak}; if needed, the workload was slightly adjusted. After verifying the workload, the apparatus was removed until a second calorimetry measurement between 15–20 min. Blood was sampled every 5 min throughout the entire protocol, as was HR and RPE. After 30 min, the workload was reduced to zero watts for a 5 min “cool down” during which subjects continued to pedal at a slow pace. After the “cool down” period between 30–35 min, participants moved to a chair where they rested for 15 min (between 35–50 min of the protocol). They continued to have blood drawn and HR measured every 5 min throughout. Blood was collected into EDTA Vacutainers (Becton-Dickinson), placed in ice, centrifuged for plasma that was transferred to −80°C. During Test Week 2, the same regimen was applied, but with the workload on the cycle ergometer matching the individual’s Test Week 1 workload to ensure equal muscle work at each of the time periods.

Weight Loss and Fitness Regimen

Subjects were prescribed a self-selected calorie-restricted diet based on the DGs and using the DRI equation to target a 10% body mass loss over 14 weeks (ca. 500–600 kcal/day reduction). A Baecke physical activity questionnaire (Baecke *et al.*, 1982) was administered to assess self-reported physical activity level with a score of 5 for the lowest activity and 15 for the highest activity related to work, sport/exercise, and non-sport leisure categories; we used a score of 7 for calculating maintenance calories. Participants recorded daily food intake in diaries and received weekly counseling from a registered dietitian. Subjects were provided with a daily nutritional supplement (Bayer One-a-Day for Women) to assure adequate intake of essential vitamins and minerals. Body mass was measured weekly. Total length of time for the weight loss and fitness intervention phase of the study varied from 14–17 wk, necessitated to match Test Weeks with respect to menstrual phase. As previously reported, we found no association of menstrual phase or blood concentrations of leutinizing or follicle-stimulating hormone with glucose homeostasis and insulin sensitivity indices (Campbell *et al.*, 2014).

Participants engaged in a prescribed exercise regimen a minimum of 4 times/wk for the duration of the intervention as directed by WHNRC exercise physiologists. Over the first 4 intervention weeks, participants exercised aerobically 4 days/wk for 30 min each (treadmill

or cycle ergometer) at an intensity of 60–70% of their maximal HR as determined in the VO_{2peak} test. During intervention weeks 5–8, exercise sessions were increased to 40 min/session, 4 days/wk and during intervention wks 9 onward the intensity was increased to a HR of 75% of maximal. Participants wore HR monitors during all exercise sessions to ensure that they were exercising at the appropriate intensity for the prescribed amount of time, with digital information downloaded by PSL staff weekly to ensure compliance.

Acylcarnitine Analysis

The details of the HPLC-MS acylcarnitine metabolite detection and analysis are presented elsewhere (Minkler *et al.*, 2015b, a). Briefly, to 10 μL of sample (plus internal standards) was added organic solvents to precipitate salts and proteins. The resulting supernatant was then applied to a mixed-mode, reversed-phase/strong cation-exchange solid-phase extraction plate (Oasis MCX, purchased from Waters Corporation, Milford, MA). Carnitine and acylcarnitines were eluted, evaporated, and derivatized with pentafluorophenacyl trifluoromethanesulfonate, then injected into a UHPLC-MS/MS system. Using sequential ion-exchange/reversed-phase chromatography, carnitine and γ-butyrobetaine were eluted in 4 min chromatograms; optimized MRM transitions were collected for carnitine, d3-carnitine internal standard, butyrobetaine, and d3-butyrobetaine internal standard. Acylcarnitines were eluted in 14 min chromatograms, and optimized MRM transitions were collected for 67 acylcarnitine species and 12 acylcarnitine internal standards. Accurately standardized calibrants were used to construct multiple-point calibration curves for accurate, absolute quantitation. Low, medium, and high quality control samples were used to validate that the analytical accuracy of each determination was within ± 15% of the actual value for medium and high values, and within ± 20% for the lowest values.

Assessment of Acylcarnitine and Metabolite Effects in Dorsal Root Ganglia (DRG) Neurons

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Utah, and conducted in the Light laboratory. Detailed experimental procedures have been described previously (Light *et al.*, 2008). In brief, muscle-innervating DRG neurons were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes, D-282). Injections of DiI were made in 7–10 d old male C57/BL6J mice (n=37 across all preparations) anesthetized by ice cold water, after which gastrocnemius, plantaris, tibialis anterior, biceps femoris, and in some mice, semitendinosus muscles were exposed. Each muscle was carefully injected with 1–3 μL DiI diluted in dimethyl formamide (J.T. Baker, 9221-01). Animals were then returned to their home cages. Around two weeks after the DiI injection, animals were euthanized with overdose of isoflurane followed by decapitation. Lumbar DRGs ipsilateral to the DiI injection sides were collected into room temperature Hank's balanced salt solution, digested with 0.25% trypsin (Sigma, #T0303), and triturated in culture medium comprised of MEM (Invitrogen, #51200-038) and 10% FBS (Hyclone). Cell suspensions were pre-plated for 1 h to reduce non-neuronal cells, and then plated onto four center wells of a 24-well plate pre-treated with poly-L-lysine (Sigma, P1274) and laminin (BD Biosciences 254232). After an initial incubation for 45 min, wells were filled with 1 mL 37°C culture medium containing 10 ng/ml glial-derived neurotrophic factor (Preprotech, 450-10) and returned to the incubator for 15–20 h before imaging experiments.

For calcium imaging, cultured DRG cells were loaded with 2 μM Fura-2 AM fluorescent dye (Molecular Probes, F1221) in culture medium for 45 min and then washed with pH 7.4 oxygenized observation medium containing no additional ATP or lactate (145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM citrate, 10 mM glucose, 10 mM MES, 10 mM HEPES). Final volume in each well was 500 μL. Cells were imaged at room temperature using the Meta Imaging Series Metafluor program (Universal Imaging). Across all experiments, each imaging field had 149 ± 3 cells, 27.5 ± 1.5% of which were labeled in retrograde fashion with DiI. These DiI-labeled cells were visualized by their fluorescent emission to 546 nm excitation. All the DiI-labeled and non-labeled cells in the fields were imaged and outlined, and their changes in fluorescence intensities (340 nm/380 nm, normalized to a range from 0 to 1) were traced every 2 sec during the experiments. At the end of each experiment, cells were treated with 200 nM capsaicin, followed by a wash with observation medium, and then a wash with 50 mM KCl. Those responding to KCl are defined as active, live neurons and those positive for a capsaicin response defined as TRPV1 receptor-positive neurons. Acylcarnitines and other metabolites were delivered transiently to the cells using a two-pipette method, with one pipette extracting solution out of the well, and another immediately adding new solution back (Light *et al.*, 2008). The pH 7.4 medium represents a “resting” level of the metabolites, and the pH 6.6 medium activates most metabolite-responsive neurons (Light *et al.*, 2008). The C16- and C6-acylcarnitines were aliquoted in DRG observation medium at 10 mM and kept frozen at −80°C; stocks were diluted to 500 μM working stocks at the time of the experiment and then added to pH 7.4 medium to the desired experimental concentrations, as indicated.

All images and data were saved for off-line replay and analysis, as described previously (Light *et al.*, 2008). Only responses of DiI-labeled neurons were analyzed, as they represented muscle-innervating cells. Off-line data were loaded into a custom-made LabVIEW (National Instruments)-based ratiometric program for further analysis. The program subtracted baseline variations, computed baseline means and SDs, and compared them with means and SDs during each metabolite addition. A baseline was 30–60 s of imaging prior to the addition of the metabolites. Responses were defined as increases of Fura ratios > 2 SDs above the baseline. From each animal, we cultured 4 wells of DRG cells. Sample sizes for each treatment, as indicated in the figure legends, represent independent wells.

Statistics and Analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software). For pre- versus post-intervention changes in exercise-associated plasma acylcarnitine concentration, a two-way ANOVA was utilized to consider effects of intervention, exercise time, and exercise X time interactions. Neuron cell-based studies that tested effects of acylcarnitines versus vehicle controls, within chain length, were analyzed by Kruskal-Wallis test due to non-normality of datasets. In neuron studies examining effect of acylcarnitines at various pH values, a two-way ANOVA was used to compare the effect of pH, acylcarnitine treatments, and interactions. A p value < 0.05 was considered to be statistically significant. Values are expressed as mean ± SEM.

Results

Body Mass loss and improvements in metabolic health indices following a fitness and dieting intervention

We reported previously (Campbell *et al.*, 2014) that the fitness and weight loss intervention led to significant reductions in body weight and adiposity, with expected marked improvements of fitness (VO_{2peak}) and substantially improved insulin sensitivity (Matsuda index). As planned, weight and a strict diet were maintained during each of the Test Weeks (Campbell *et al.*, 2014). Several phenotype parameters related to fitness and exercise fuel combustion are presented for context (Table 1).

1. Overnight-fasted plasma acylcarnitine concentrations following weight loss and fitness intervention

Values for all individual plasma acylcarnitine concentrations in sedentary overnight-fasted insulin resistant subjects (pre-intervention) and in the subjects after becoming metabolically fit and insulin sensitive (post-intervention) are provided in Table 2. Despite significant improvements in metabolic health, there were few differences in fasting plasma acylcarnitine profiles. For instance, there were no significant differences in fasting plasma free carnitine concentration or in plasma acetylcarnitine, a metabolite derived from catabolism of all fuels (fatty acids, some amino acids, and pyruvate) that yield acetyl-CoA. Interestingly, there was a significant effect of fitness and weight loss intervention to increase fasting plasma concentration of the carnitine precursor γ-butyrobetaine, when evaluated by 2-way ANOVA (Figure 1); the reduction in plasma cis-3,4-heptanoylcarnitine following intervention was also significant when evaluated as a 2-way ANOVA (Figure 4). Fasting plasma acylcarnitines associated with fatty acid and amino acid metabolism will be discussed in succession.

Fatty acid metabolism and incomplete long-chain fatty acid β-oxidation—We hypothesized that overnight-fasted plasma concentrations of medium-chain acylcarnitines, indices of incomplete LCFA β-oxidation, would be reduced in the more insulin-sensitive, metabolically-fit state following intervention. However, fasting C6- to C14 acylcarnitines remained essentially unchanged by the intervention (Table 2 and Figure 2). The same lack of intervention effect was observed for the short-chain acyl-CoA derivative butyrylcarnitine (Table 2 and Figure 2), as well as hydroxy-derivatives of MCFA and LCFA that mark incomplete mitochondrial β-oxidation (Table 2). In distinct contrast to other MCFA-carnitines, intervention was associated with a reduction in concentration of cis-3,4-methylene-heptanoylcarnitine (Figure 4). Overnight-fasting plasma concentration of a similar metabolite, cis-3,4-methylene-nonanoylcarnitine, was not significantly impacted by intervention (Table 2 and Figure 4). Notably, the parent LCFA (cis-13,14-methylene-heptadecanoic acid), from which cis-3,4-methylene-heptanoylcarnitine is ultimately derived following partial β-oxidation, appears to be a xenometabolite produced by gut microbes (Libert *et al.*, 2005; Yang *et al.*, 2007).

Overnight-fasted plasma concentrations of long-chain saturated and unsaturated acylcarnitines (C16 chain length and above) were almost all reduced in the post-intervention

state (Table 2). Most apparent were the significant reductions in concentrations of palmitoylcarnitine (C16; $p<0.05$, paired t-test) and oleoylcarnitine (C18:1; $p = 0.05$, paired t-test). There was a significant intervention effect for palmitoyl-, oleoyl- and linoleoylcarnitine (C18:2) (Figure 5). These observations are suggestive of reduced fasting plasma LCFAs that ultimately provide LCFA-CoA substrate for tissue carnitine palmitoytransferase 1 (CPT1). Consistent with this perspective, overnight-fasted plasma LCFA concentrations were modestly but consistently lower following intervention in this cohort (Supplemental Figure 2).

Amino acid metabolism—Perturbations in BCAA homeostasis as reflected in higher blood BCAA concentrations are evident with deteriorating insulin sensitivity and blood sugar control (reviewed in (Adams, 2011; Lynch & Adams, 2014)); thus, overnight-fasted plasma concentrations of acylcarnitine derivatives of BCAA catabolism were determined. It was anticipated that improved metabolic health following weight loss and fitness intervention would alter acylcarnitine markers of BCAA in a manner suggestive of more complete or efficient catabolism, downstream of the BCAA oxidation check-point mitochondrial branched chain ketoacid dehydrogenase complex (BCKDC). However, there were no obvious differences comparing the pre- vs. post-intervention phases with respect to overnight-fasted plasma concentrations of carnitine derivatives of leucine (isovaleryl-, 3-methylcrotonyl- or 3-methylglutaroyl-, 3-hydroxyisovaleryl-) or isoleucine (2-methylbutyryl-, tigloyl-, methylmalonyl-, propionyl-) (Table 2). There was a significant intervention effect for one valine derivative (isobutyryl-), which was modestly increased in overnight-fasted plasma with improved fitness and weight loss (Figure 7, **left panels**). Isovaleryl-, 2-methylbutyryl- and isobutyrylcarnitines (Figure 7) are derived from CoA metabolites immediately downstream of the BCKDC.

2. Exercise-associated plasma acylcarnitine patterns

Plasma concentration graphs for all detectable plasma acylcarnitine metabolites are provided in Supplemental Figure 1. For brevity, only a subset of acylcarnitines associated with LCFA β-oxidation and BCAA catabolism are presented and discussed. Data are expressed both as absolute concentrations and as a change from the basal, pre-exercise value using each subject's time zero value as 100%. The latter led to substantially less variance in the data, allowing for clear interpretation of intervention- and exercise-associated changes in acylcarnitine metabolism. As anticipated, a sub-maximal exercise bout designed to engage muscle LCFA oxidation led to significant shifts in a variety of plasma acylcarnitines, reflective of temporal changes in mitochondrial fuel metabolism.

Free carnitine, acetyl-carnitine, and γ-butyrobetaine—Exercise led to an acute reduction in plasma free carnitine concentration in both the pre- and post-intervention condition, most clearly illustrated in the temporal pattern as a percent of pre-exercise concentration (Figure 1). This likely reflects utilization of this metabolite to generate acylcarnitine moieties derived from enhanced fuel catabolism and concurrent flux to acyl-CoA metabolites. Free carnitine continued to fall during the post-exercise cool-down period that began following the 30 min sample collection. Plasma acetylcarnitine rose significantly with exercise, and continued to increase even with exercise cessation (Figure 1). As the most

abundant acylcarnitine, the rise in acetylcarnitine likely contributed to the concurrent reduction in free carnitine. Plasma concentrations of the carnitine precursor γ-butyrobetaine were reduced acutely by exercise and its pattern generally was similar to free carnitine concentration (Figure 1). The temporal patterns for these metabolites did not differ when comparing pre- and post-intervention conditions, but as noted previously there was a significant intervention effect for γ-butyrobetaine.

Fatty acid metabolism and incomplete long-chain fatty acid β-oxidation— Exercise led to a rapid and significant increase in plasma concentrations of chain-shortened products of incomplete LCFA β-oxidation, including C6- to C14-acylcarnitines derived from saturated LCFA catabolism (Figures 2 and 3). In addition, chain-shortened products of incomplete unsaturated LCFA combustion, such as decenoylcarnitine derivatives, were also generally increased by exercise (Supplemental Figure 1). Butyrylcarnitine (C4) concentrations were also increased by an acute exercise bout (Figures 2 and 3). The exercise-increased concentrations of the plasma metabolites cis-3,4-methylene-heptanoylcarnitine and cis-3,4-methylene-nonanoylcarnitine are shown in Figure 4. Altogether, these results indicate that changes in most blood MCFA-carnitines were yoked to acute changes in muscle work. Exercise cool-down was accompanied by progressive—sometimes sharp—reductions in these metabolites (i.e., in samples taken at 35 min onward). Notably, plasma concentrations of C4-C12 MCFA-carnitines remained high and stable during the initial 5 min post-exercise period, before falling thereafter. A post-exercise reduction in plasma C14-carnitine was not clearly demonstrated.

Plasma LCFA-carnitines were increased by the acute exercise bout (Figures 5–6), but in contrast to MCFA-carnitines, the exercise-associated rise was most clearly apparent only in the post-intervention condition (Figure 6). Furthermore, upon cessation of exercise, concentrations of most LCFA-carnitines tended to remain elevated or have a slow downward trajectory. The contrasting exercise-associated patterns comparing pre- and post-intervention suggest that extra-mitochondrial metabolism of LCFA—but not intra-mitochondrial FAO—was altered by the weight loss and improved fitness.

BCAA metabolism—Patterns of BCAA pathway carnitine derivatives stemming from acyl-CoA metabolites produced by the BCKDC suggest an acute reduction in BCAA catabolism during the initial phase of exercise (Figure 7). The metabolites 2-methylbutyrylcarnitine (from Ile), isovalerylcarnitine (from Leu), and isobutyrylcarnitine (from Val) were all reduced over the 10–15 exercise bout time frame, after which levels tended to recover. There was no clear effect of fitness and weight loss intervention on the temporal acute exercise effect. The exercise bout had little effect on BCAA-metabolites that are downstream of these metabolites, e.g., tigloyl-carnitine (from 2-methylbutyryl-CoA-derived tigloyl-CoA), or 3-methyl-crotonyl-carnitine, 3-methyl-glutaroyl-carnitine, 3-hydroxy-isovalerylcarnitine (from CoA metabolites downstream of isovaleryl-CoA): see Supplemental Figure 1.

3. Proof-of-Principle Study: Can acylcarnitines serve as signals for somatosensory neurons innervating skeletal muscle?

We and others have shown that select acylcarnitines have a variety of cellular bioactivities that may involve increases in intracellular calcium, modulation of protein kinase C, activation of caspase-3 and regulation of ion channels (McCoin *et al.*, 2015a). Since plasma concentrations illustrated that acylcarnitine production is increased with acute exercise, we hypothesize they—singly or in combination with other metabolites—serve as exertion-sensitive signals to the nerves that interrogate the muscle. As an initial exploration of this hypothesis, the actions of a medium-chain acylcarnitine (C6-carnitine) and long-chain acylcarnitine (C16-carnitine) on activation of somatosensory neurons associated with mouse muscle were tested. DiI-positive neurons isolated from the dorsal root ganglia (DRG) clearly marked specific neuronal populations innervating muscle that had previously been injected with this dye (Figure 8A). These specific neurons were subsequently tracked with respect to activation (rise in intracellular calcium) in response to acylcarnitines applied in the cell bath system, as illustrated in Figure 8B.

Palmitoylcarnitine at concentrations of 1–10 μM activated a small sub-population of DRG neurons (~2.5–5.5%, depending on the experiment)(Figure 8C). This effect became most apparent, and statistically significant, at a concentration of 10 μM. At higher concentrations, the percent of cells that were activated by C16-carnitine was substantially increased to ~30% at 25 μM and 80% at 50 μM (Figure 8C). At all concentrations tested, C6-carnitine had little to no effect on activation of DRG neurons, in contrast to C16-carnitine (Figure 8C). Considering that exercise-associated signals to muscle-innervating neurons likely involve a complex milieu of metabolites, acylcarnitines were also applied in the presence of lactate and ATP at concentrations and pH conditions similar to that previously used by the Light laboratory to “mimic” exercise (Light *et al.*, 2008). In the presence of lactate and ATP alone, neuron activation was triggered and as previously reported, and this increased significantly at lower pH ranges (Figure 8D). The presence of acylcarnitines at 10 μM did not further augment this response, and the increase in activated neurons in response to acylcarnitines at the pH 6.6 condition did not reach statistical significance. In summary, C16-carnitine led to increases in intracellular calcium in a small sub-set of muscle-innervating DRG neurons *ex vivo* at the lowest concentrations tested, but C6- or C16-carnitine did not increase the responses of DRG neurons caused by lactate or ATP under the conditions tested.

Discussion

There is strong evidence that insulin resistance and T2DM are associated with altered mitochondrial fuel management. For instance, these conditions are often associated with mismatched TCA cycle activity relative to increased fatty acid fuel delivery, leading to incomplete FAO in muscle cells (Koves *et al.*, 2008; Aguer *et al.*, 2015) and tissue and blood accumulation of lipid intermediates such as acylcarnitines (Genuth & Hoppel, 1981; Inokuchi *et al.*, 1995; Moder *et al.*, 2003; Koves *et al.*, 2008; Adams *et al.*, 2009; Mihalik *et al.*, 2010). Other lipid derivatives, such as diacylglycerols (DAGs) and ceramides can also build up and may exacerbate insulin resistance in tissues such as liver and muscle (Amati, 2012; Chaurasia & Summers, 2015). Based on pro-inflammatory actions of chain-shortened

acylcarnitines we observed in pilot studies (Adams *et al.*, 2009), and in light of literature indicating that blockade of CPT-1 ameliorates cultured myocyte insulin resistance (Koves *et al.*, 2008), we proposed the hypothesis that acylcarnitine metabolites are involved with cell stress and inflammation responses associated with insulin resistance (Adams *et al.*, 2009). This concept has been supported by experimental evidence in immune and muscle cell culture studies (Rutkowsky *et al.*, 2014; Aguer *et al.*, 2015), adding to the historic literature suggesting bioactivities of select acylcarnitines (McCoin *et al.*, 2015a). Thus, understanding the basis of incomplete FAO and its potential consequences, and the potential roles of acylcarnitines as signaling or regulatory metabolites, has important implications for metabolic health.

Contributors to the FAO/TCA cycle mismatch in insulin resistance and T2DM may be a reduced number of muscle mitochondria and/or lower complete combustion (i.e., (He *et al.*, 2001; Kelley *et al.*, 2002; Petersen *et al.*, 2004; Morino *et al.*, 2005; Ritov *et al.*, 2005; Befroy *et al.*, 2007; Mogensen *et al.*, 2007; Schrauwen & Hesselink, 2008)). Yet, the assertion that mitochondrial dysfunction underlies diabetes phenotypes has been questioned, because isolated mitochondria from insulin-resistant or T2DM individuals can display normal bioenergetics and the muscle is calculated to have oxidative capacity that far exceeds what is required for maximal ATP generation (see (Holloszy, 2009)). Reconciling these various perspectives must take into account several key principles: (1) That studies using isolated mitochondria are provided fuel and anaplerotic TCA substrates artificially, potentially masking intrinsic perturbations of TCA activity that occur *in situ* due to “anaplerotic/cataplerotic stress” (Adams *et al.*, 2009; Fiehn *et al.*, 2010; Zoppi *et al.*, 2010); (2) Meeting muscle ATP needs through FAO can take place without full combustion of the fat in the TCA cycle: for any given ATP requirement, one may generate chain-shortened fatty acid metabolites through β-oxidation that do not enter the TCA cycle at all, but simply accumulate or are exported from mitochondria; (3) While measuring muscle fiber metabolism *ex vivo* is an improvement over isolated mitochondria, bioenergetics profiles comparing insulin-resistant and insulin-sensitive preparations will still be strongly influenced by the ATP turnover requirements of the cells, which may differ. With these concepts in mind, we set out to determine if incomplete LCFA combustion (*viz.* matching of fuel delivery to TCA activity) is ameliorated *in vivo* concurrent with robust increases in metabolic fitness and insulin sensitivity in previously-sedentary, obese, insulin-resistant women. Secondarily, acylcarnitine patterns related to BCAA catabolism were determined since blood BCAA concentrations correlate to insulin resistance phenotypes and oxidation of BCAA is regulated in part by FAO (Adams, 2011; Lynch & Adams, 2014). A sub-maximal exercise bout and duration designed to trigger muscle work and fat oxidation was leveraged to address these questions.

The first important finding from these studies is that despite marked improvements in insulin sensitivity and fitness, there was no evidence for a difference in the “completeness” of fasting or exercise-induced intramitochondrial FAO–as defined by plasma concentrations and temporal responses of short- and medium-chain acylcarnitines. This was surprising considering the large body of evidence indicating that in the T2DM or insulin resistant states, circulating levels of many acylcarnitines including chain-shortened moieties are higher (Genuth & Hoppel, 1981; Inokuchi *et al.*, 1995; Moder *et al.*, 2003; Koves *et al.*,

2008; Adams *et al.*, 2009; Mihalik *et al.*, 2010), and incomplete FAO is observed even in cultured myotubes derived from insulin resistant models (Koves *et al.*, 2008; Aguer *et al.*, 2015). Our observations regarding no intervention effects on overnight-fasted, resting plasma medium-chain acylcarnitines are similar to the report of Rodriguez-Gutierrez who studied effects of 10 wk aerobic exercise intervention in overweight adults (Rodriguez-Gutierrez *et al.*, 2012). Resting muscle biopsy results from trained (presumably exquisitely insulin-sensitive) individuals displayed increased, not decreased, acylcarnitine content across chain lengths (Huffman *et al.*, 2014). Together, these published results and ours herein indicate that factors beyond insulin resistance dictate tissue and blood acylcarnitine patterns.

With respect to the metabolic shifts inherent to an acute exercise bout, as expected blood concentrations of all chain-shortened acylcarnitines were significantly and rapidly increased, indicative of accelerated FAO. In the case of C4- through C12-carnitines, cessation of exercise led to a rapid drop in their concentrations, as also described by others (i.e., (Lehmann *et al.*, 2010; Hansen *et al.*, 2015)). Assuming that during vigorous sub-maximal exercise the acute changes in blood acylcarnitine patterns track muscle accumulation (Hiatt *et al.*, 1989; Friolet *et al.*, 1994), we interpret these plasma patterns to indicate that (a) regardless of insulin sensitivity and fitness status in non-diabetic women, muscle intramitochondrial β-oxidative flux is being driven wholly by ATP turnover (oxidative phosphorylation), and (b) this process is inherently inefficient in that chain-shortened acylcarnitines rise dramatically with exercise, independent of improvements in fitness and insulin sensitivity. The latter aligns with observations comparing T2DM and non-diabetic subjects during exercise (Hansen *et al.*, 2015). Teleologically, inefficient FAO during exercise enables rapid flux through β-oxidation to meet increased ATP needs, with acylcarnitine generation supporting continued availability of the finite intramitochondrial CoASH pools that support oxidative metabolism. We conclude that under conditions in which muscle work is held constant, intramitochondrial FAO and the attendant generation of chain-shortened acyl-CoA moieties (reflected in acylcarnitine patterns) are directly tethered to total muscle oxidative phosphorylation (ATP turnover), independent of tissue or whole-body metabolic health. A caveat to this interpretation is that in our fixed workload model, metabolism was sampled in the post-intervention trained subjects at a lower percent of their VO_{2peak} compared to the pre-intervention state. This would have been accompanied by a greater reliance on fat metabolism to meet ATP needs in the post-intervention phase when fitness was significantly improved, as per the “crossover” concept of Brooks and Mercier (Brooks & Mercier, 1994), and as illustrated by the modest intervention-associated increase in average exercise bout FAO (14% higher) and reduced carbohydrate oxidation (17% lower) in our subjects. A limitation of the current study is that tracer kinetics or other methods were not used to track tissue-specific and whole-body FAO with a higher degree of definition compared to indirect calorimetry during the dynamic state of acute exercise. Furthermore, while the tight recruitment criteria and controlled nature of the experiments and diets were robust, the small sample size of the cohort may have dampened statistical power to detect stronger intervention-associated differences in fuel choice during exercise.

In contrast to the equivalent patterns of short- and medium-chain acylcarnitines in pre- vs. post-intervention conditions, plasma long-chain acylcarnitines (relative to resting, overnight-fasted levels) were significantly impacted by intervention. Post-intervention, long-chain

acylcarnitines increased with acute exercise, whereas pre-intervention exercise-induced responses for LCFA-carnitines were weak, in many cases remaining flat or modestly and slowly increased. This was not anticipated. We suspect that the increased LCFA-carnitine exercise-associated trajectories following fitness and weight loss intervention relates to training effects that would have increased *in situ* muscle CPT1 activity. CPT1 abundance has been reported to be ~25% higher in muscle of endurance-trained vs. sedentary individuals (Jong-Yeon *et al.*, 2002), and muscle activity/expression of malonyl-CoA decarboxylase (an enzyme that degrades the CPT1 inhibitor malonyl-CoA) is increased by training (Kuhl *et al.*, 2006). Furthermore, training leads to an increase in muscle mitochondrial capacity (i.e., (Short *et al.*, 2003; Menshikova *et al.*, 2005; Menshikova *et al.*, 2007)), and by extension this would yield a higher whole-tissue, resident CPT1 capacity. Unlike the enzymes that control intramitochondrial β-oxidation that generate chain-shortened acyl-CoAs and their acylcarnitine derivatives, CPT1 is not regulated by oxidative phosphorylation and attendant changes in the intramitochondrial NAD^+/NADH status. The sharp reduction in plasma LCFA with acute exercise (Supplemental Figure 2) is consistent with enhanced utilization of this substrate from the blood pool. In the face of increased LCFA delivery to exercising muscle, higher whole-muscle CPT1 activity following a weight loss and training intervention would drive greater increases in long-chain acylcarnitine generation and export, as suggested by the results herein. The postulate that intervention-associated increases in exercise-induced LCFA-carnitines reflect the muscle bed CPT1 activity will require additional biopsy-based studies to validate. Interestingly, in the overnight-fasted, resting state there was a significant reduction in the concentration of several LCFA-carnitines following the weight loss and training intervention. The physiological ramifications of intervention effects on resting- and exercise-associated plasma LCFA-carnitines are not clear. The possibility that differential LCFA-carnitine myocyte and mitochondrial accumulation influences muscle cell stress, myokine production (i.e., interleukin-6 [IL-6]), ion transporters and other activities is worth considering (McCoin *et al.*, 2015a; McCoin *et al.*, 2015b). Long-chain acylcarnitines and other exercise-associated metabolites—singly or in combination—also have the potential to serve as signaling metabolites to neurons communication information from the muscle bed, as discussed below.

In addition to fatty acid metabolism, acylcarnitine profiling provided insights as to the mitochondrial catabolism of BCAA fuels during exercise and comparing pre- vs. post-fitness and weight loss intervention. Of particular interest is the question of whether or not improvements in metabolic health lead to changes in branched chain amino acid (BCAA) metabolism, since modest elevations in fasting blood BCAA concentrations track and predict insulin resistance and T2DM risk (reviewed in: (Adams, 2011; Lynch & Adams, 2014)). In the current experiment, plasma carnitine derivatives of BCAA-derived CoA metabolites just downstream of the rate-limiting enzyme complex in the mitochondrial BCAA catabolism pathway (BCKDC) were largely unaffected by the training and weight loss intervention, despite marked improvement in insulin sensitivity. These data are consistent with BCAA metabolite profiling in this cohort (Campbell *et al.*, 2014) and in a separate weight loss cohort (Piccolo *et al.*, 2015): in those studies, there was no evidence from plasma BCAA or branched chain ketoacid (BCKA) patterns that intervention-associated improvements in insulin sensitivity altered BCAA catabolism pathways. Indeed,

the commonly-reported correlation between blood BCAA and insulin resistance indices seems to be lost upon weight loss intervention (Petersen *et al.*, 2012; Piccolo *et al.*, 2015). Thus, the results highlight once again that the link between insulin resistance and circulating BCAA concentrations (and perhaps BCKDC) are context-specific. It is notable that there was an intervention effect for the valine-derived metabolite isobutyrylcarnitine, which was increased by the weight loss and training intervention. The basis for this unique profile is not clear but suggests differential catabolic flux of valine. From a technical standpoint, it is worth noting that the acylcarnitine analytical methods used herein are superior to tandem MS since isobutyrylcarnitine and butrylcarnitine could be distinguished; these metabolites changed in opposite directions during the exercise bout, underscoring that they each have different fuel precursors. Recently, elegant studies by Arany et al. pointed to 3-hydroxyisobutyrate (3-HIB)—a metabolite downstream of isobutryl-CoA—as a paracrine factor promoting muscle fatty acid uptake and insulin resistance (Jang *et al.*, 2016). Our results showing no effect of improved metabolic fitness and insulin sensitivity on plasma leucine and isoleucine acylcarnitine derivatives, and an increase in a valine catabolic derivative under these conditions, highlights the equivocal nature of the BCAA-insulin resistance association.

A second important finding related to BCAA catabolism was that exercise-induced temporal patterns of acylcarnitines reflective of BCKDC activity differed among the specific BCAA derivatives. For instance, concentration of the leucine/2-ketoisocaproate derivative isovalerylcarnitine was rapidly reduced with acute exercise, and the isoleucine/2-keto-methylvalerate derivative 2-methylbutyrylcarnitine displayed a similar reduction but less marked and more delayed. The transient drop in these metabolites likely reflects acute inhibition of the BCKDC by increased FAO ((Corkey *et al.*, 1982); also see (Adams, 2011)). Concentrations of these metabolites recovered partially or completely as exercise continued, and the valine/2-ketoisovalerate derivative isobutyrylcarnitine was variably impacted by exercise. It is not immediately clear why there are differing patterns of metabolites associated with each of the individual BCAA: differences in availability of substrate BCKAs, or differing biochemical activities of the specific enzymes downstream of BCKDC *in situ*, may be considered. Overall, we conclude that the BCAA derivative acylcarnitine patterns support the idea that activation of FAO acutely down-regulates oxidative flux of BCAA during an exercise bout.

An exciting finding was that the weight loss and training intervention led to significant changes in the concentration of metabolites associated with gut microbiome metabolism (“non-self” xenometabolites). In other words, host health impacted gut microbiome metabolism, as we have highlighted in a previous paper stemming from this cohort (Campbell *et al.*, 2014). Extending those results, in the current study plasma *cis*-3,4-methylene-heptanoylcarnitine was significantly reduced in the post-intervention condition: this metabolite is a medium-chain derivative of a long-chain fatty acid that is almost certainly a xenometabolite. Acylcarnitine fatty acid metabolites with a cyclopropane ring reportedly disappeared in the urine of humans treated with the antibiotic adriamycin, and were not detectable in urine from newborns (Libert *et al.*, 2005), suggestive of a gut microbe origin for the parent long-chain acyl group. It is proposed that cis-3,4-methylene-heptanoylcarnitine emanates from incomplete β-oxidation of *cis*-13,14-methylene-

heptadecanoic acid, a product of bacterial metabolism (also see (Yang *et al.*, 2007)). A strength of the current study is that metabolomics evaluations were conducted under a controlled feeding paradigm in which individuals were fed the exact same diets (Campbell *et al.*, 2014). These results support the concept that improved metabolic health and weight loss modify the gut microbe ecology and metabolism through host-derived signals independent of diet (Campbell *et al.*, 2014). We also observed an intervention-associated increase in the co-metabolite (having potentially both microbial and host origin) γ-butyrobetaine, a carnitine pathway intermediate whose circulating concentrations can be impacted by gut microbial metabolism of dietary carnitine (Koeth *et al.*, 2014). However, the idea that intervention-associated changes in this metabolite were due to microbiome alterations is tempered by the fact that γ-butyrobetaine is also be derived from endogenous enzyme activities, starting with trimethyllysine made available through protein breakdown/turnover (Hoppel & Davis, 1986). Further research is warranted to explore how host metabolic health influences gut microbe metabolism and the resulting xenometabolome, to elucidate how these metabolites are stored and transported in the body, and to understand which xenometabolites display tissue bioactivities.

A final set of observations involved the question of whether or not muscle metabolites serve as exertion signals to somatosensory afferent neurons that interrogate muscle beds. If true, such factors could inform the CNS and/or motor neurons as to muscle workload, fuel homeostasis, fatigue and muscle condition. As an initial evaluation of acylcarnitines as candidate exertion signals, we conducted proof-of-principle studies looking at effects of C6- (medium-chain) and C16-carnitine (long-chain) metabolites on activation of muscle-innervating mouse dorsal root ganglia neurons. In the absence of lactate and ATP metabolites known to trigger these cells (Light *et al.*, 2008), 1–10 μM palmitoylcarnitine activated a small subset of neurons (~2.5–5.5%) as indicated by increased intracellular Ca^{2+}, in contrast to hexanoylcarnitine that had little to no effect at any concentration. We speculate that the very high activation rates observed at 25–50 μM C16-carnitine were due to perturbations in the neuronal membranes due to the zwitterionic nature of this metabolite (McCoin *et al.*, 2015a). The biochemical milieu surrounding muscle during exercise does not consist of single metabolites in isolation, which may explain the modest activation at potentially physiological levels (C16-carnitine) or lack thereof at any concentration in the case of C6-carnitine. A mixture of metabolites and changes in pH used previously to model the muscle biochemistry of modest to significant exertion (Light *et al.*, 2008) activated DRG neurons, with increasing populations of cells engaged as pH was lowered into ranges (mid-6s) typically reported during modest to exhaustive exercise (Sahlin *et al.*, 1976; Costill *et al.*, 1982; Katz *et al.*, 1984). This effect was not clearly augmented by the presence of acylcarnitines under the current conditions. We cannot fully exclude the possibility that C16-carnitine effects on neurons were due to provision of metabolizable energy; however, countering this perspective is the fact that C6-carnitine did not trigger DRG neurons, and only a small subset of neurons were responsive at lower concentrations of C16-carnitine which suggests specificity. Certainly, the *ex vivo* neuron results using C16-carnitine singly–coupled to the close association between muscle work and acylcarnitine production–support the further study of acylcarnitines and other muscle-derived metabolites as fatigue or exertion signals involved in a muscle-somatosensory-CNS communication system. Such

studies should also consider additional chain-lengths including more abundant acylcarnitines. The potential that such a system impacts the muscle-somatosensory-motor neuron short-loop is also intriguing to consider. Since the concentrations of acylcarnitines *circa* the muscle-neuron interface are not yet known, the physiological relevance of the current observations remains a question to be answered in future experiments.

In summary, we have performed the most comprehensive assessment to date of human blood acylcarnitine patterns in response to acute exercise and a weight loss/training intervention, during a controlled feeding period designed to minimize diet-associated influence on the metabolome and microbiome. The results highlight that improved metabolic health failed to alter markers of incomplete LCFA β-oxidation at rest or during a standard exercise bout that engaged equivalent levels of muscle work. This strongly suggests that that intramitochondrial generation of chain-shortened acylcarnitines in exercising muscle is tethered closely to the absolute amount of oxidative phosphorylation (ATP turnover). Furthermore, we confirmed prior observations in this group of women that weight loss, improved fitness and insulin sensitivity alters xenometabolites or their derivatives in the body, indicating that host metabolic status drives changes in gut microbe metabolism, even under controlled feeding conditions. Finally, a proof-of-principle study revealed an exciting new finding; namely, that palmitoylcarnitine can activate muscle-innervating somatosensory afferents. This raises the intriguing possibility that myocyte acylcarnitine and other metabolites responsive to exercise serve as signaling metabolites to neurons that inform the brain on exertion, muscle work level and duration, and fuel utilization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Mary Gustafson and the Human Studies Unit, plus the Analytical Support Lab staff of the USDA-ARS WHNRC for helping support screening-recruitment efforts, and blood collection and processing. Supported in part by USDA-ARS Projects 5306-51530-016-00D, 5306-51530-019-00 and 6026-51000-010-05S, and NIH-NIDDK R01DK078328 (SHA, CLH, OF, NLK, JWN, WTG, GRH, JRF, MEH), NIH U24DK097154 for the West Coast Metabolomics Center (OF, JWN). USDA is an equal opportunity provider and employer.

Abbreviations

CNS	central nervous system
DRG	dorsal root ganglia
TCA cycle	tricarboxylic acid cycle
FAO	fatty acid oxidation
BCAA	branched-chain amino acid
RQ	respiratory quotient
LCFA	long-chain fatty acid

T2DM	type 2 diabetes mellitus
QUICKI	Quantitative Insulin Sensitivity Check
HOMA	Homeostasis Model Assessment
RMR	resting metabolic rate
BMI	body mass index
CPT	carnitine palmitoyltransferase
TCA cycle	tricarboxylic acid cycle

References

Adams SH. Emerging perspectives on essential amino acid metabolism in obesity and the insulin-resistant state. Adv Nutr. 2011; 2:445–456. [PubMed: 22332087]

Adams SH, Hoppel CL, Lok KH, Zhao L, Wong SW, Minkler PE, Hwang DH, Newman JW, Garvey WT. Plasma acylcarnitine profiles suggest incomplete long-chain fatty acid beta-oxidation and altered tricarboxylic acid cycle activity in type 2 diabetic African-American women. J Nutr. 2009; 139:1073–1081. [PubMed: 19369366]

Aguer C, McCoin CS, Knotts TA, Thrush AB, Ono-Moore K, McPherson R, Dent R, Hwang DH, Adams SH, Harper ME. Acylcarnitines: potential implications for skeletal muscle insulin resistance. FASEB J. 2015; 29:336–345. [PubMed: 25342132]

Amati F. Revisiting the diacylglycerol-induced insulin resistance hypothesis. Obes Rev. 2012; 13(Suppl 2):40–50. [PubMed: 23107258]

Baecke JA, Burema J, Frijters JE. A short questionnaire for the measurement of habitual physical activity in epidemiological studies. Am J Clin Nutr. 1982; 36:936–942. [PubMed: 7137077]

Befroy DE, Petersen KF, Dufour S, Mason GF, de Graaf RA, Rothman DL, Shulman GI. Impaired mitochondrial substrate oxidation in muscle of insulin-resistant offspring of type 2 diabetic patients. Diabetes. 2007; 56:1376–1381. [PubMed: 17287462]

Blaak EE, van Aggel-Leijssen DP, Wagenmakers AJ, Saris WH, van Baak MA. Impaired oxidation of plasma-derived fatty acids in type 2 diabetic subjects during moderate-intensity exercise. Diabetes. 2000; 49:2102–2107. [PubMed: 11118013]

Blaak EE, Wagenmakers AJ. The fate of [U-(13)C]palmitate extracted by skeletal muscle in subjects with type 2 diabetes and control subjects. Diabetes. 2002; 51:784–789. [PubMed: 11872680]

Brooks GA. Importance of the ‘crossover’ concept in exercise metabolism. Clin Exp Pharmacol Physiol. 1997; 24:889–895. [PubMed: 9363377]

Brooks GA, Mercier J. Balance of carbohydrate and lipid utilization during exercise: the “crossover” concept. J Appl Physiol (1985). 1994; 76:2253–2261. [PubMed: 7928844]

Campbell C, Grapov D, Fiehn O, Chandler CJ, Burnett DJ, Souza EC, Casazza GA, Gustafson MB, Keim NL, Newman JW, Hunter GR, Fernandez JR, Garvey WT, Harper ME, Hoppel CL, Meissen JK, Take K, Adams SH. Improved metabolic health alters host metabolism in parallel with changes in systemic xeno-metabolites of gut origin. PLoS One. 2014; 9:e84260. [PubMed: 24416208]

Chaurasia B, Summers SA. Ceramides - Lipotoxic Inducers of Metabolic Disorders. Trends Endocrinol Metab. 2015; 26:538–550. [PubMed: 26412155]

Coen PM, Goodpaster BH. Role of intramyocelluar lipids in human health. Trends Endocrinol Metab. 2012; 23:391–398. [PubMed: 22721584]

Corkey BE, Martin-Requero A, Walajtys-Rode E, Williams RJ, Williamson JR. Regulation of the branched chain alpha-ketoacid pathway in liver. J Biol Chem. 1982; 257:9668–9676. [PubMed: 6809741]

Costill DL, Sharp RL, Fink WJ, Katz A. Determination of human muscle pH in needle-biopsy specimens. J Appl Physiol Respir Environ Exerc Physiol. 1982; 53:1310–1313. [PubMed: 7174423]

Fery F, Balasse EO. Ketone body turnover during and after exercise in overnight-fasted and starved humans. Am J Physiol. 1983; 245:E318–325. [PubMed: 6353933]

Fery F, Balasse EO. Response of ketone body metabolism to exercise during transition from postabsorptive to fasted state. Am J Physiol. 1986; 250:E495–501. [PubMed: 3518484]

Fiehn O, Garvey WT, Newman JW, Lok KH, Hoppel CL, Adams SH. Plasma metabolomic profiles reflective of glucose homeostasis in non-diabetic and type 2 diabetic obese African-American women. PLoS One. 2010; 5:e15234. [PubMed: 21170321]

Friolet R, Hoppeler H, Krahenbuhl S. Relationship between the coenzyme A and the carnitine pools in human skeletal muscle at rest and after exhaustive exercise under normoxic and acutely hypoxic conditions. J Clin Invest. 1994; 94:1490–1495. [PubMed: 7929825]

Genuth SM, Hoppel CL. Acute hormonal effects on carnitine metabolism in thin and obese subjects: responses to somatostatin, glucagon, and insulin. Metabolism. 1981; 30:393–401. [PubMed: 6111018]

Hansen JS, Zhao X, Irmler M, Liu X, Hoene M, Scheler M, Li Y, Beckers J, Hrabe de Angelis M, Haring HU, Pedersen BK, Lehmann R, Xu G, Plomgaard P, Weigert C. Type 2 diabetes alters metabolic and transcriptional signatures of glucose and amino acid metabolism during exercise and recovery. Diabetologia. 2015; 58:1845–1854. [PubMed: 26067360]

He J, Watkins S, Kelley DE. Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity. Diabetes. 2001; 50:817–823. [PubMed: 11289047]

Hiatt WR, Regensteiner JG, Wolfel EE, Ruff L, Brass EP. Carnitine and acylcarnitine metabolism during exercise in humans. Dependence on skeletal muscle metabolic state. J Clin Invest. 1989; 84:1167–1173. [PubMed: 2794054]

Holloszy JO. Skeletal muscle “mitochondrial deficiency” does not mediate insulin resistance. Am J Clin Nutr. 2009; 89:463S–466S. [PubMed: 19056574]

Hoppel CL, Davis AT. Inter-tissue relationships in the synthesis and distribution of carnitine. Biochem Soc Trans. 1986; 14:673–674. [PubMed: 3091422]

Huffman KM, Koves TR, Hubal MJ, Abouassi H, Beri N, Bateman LA, Stevens RD, Ilkayeva OR, Hoffman EP, Muoio DM, Kraus WE. Metabolite signatures of exercise training in human skeletal muscle relate to mitochondrial remodelling and cardiometabolic fitness. Diabetologia. 2014; 57:2282–2295. [PubMed: 25091629]

Inokuchi T, Imamura K, Nomura K, Nomoto K, Isogai S. Changes in carnitine metabolism with ketone body production in obese glucose-intolerant patients. Diabetes Res Clin Pract. 1995; 30:1–7. [PubMed: 8745200]

Jacob S, Machann J, Rett K, Brechtel K, Volk A, Renn W, Maerker E, Matthaei S, Schick F, Claussen CD, Haring HU. Association of increased intramyocellular lipid content with insulin resistance in lean nondiabetic offspring of type 2 diabetic subjects. Diabetes. 1999; 48:1113–1119. [PubMed: 10331418]

Jang C, Oh SF, Wada S, Rowe GC, Liu L, Chan MC, Rhee J, Hoshino A, Kim B, Ibrahim A, Baca LG, Kim E, Ghosh CC, Parikh SM, Jiang A, Chu Q, Forman DE, Lecker SH, Krishnaiah S, Rabinowitz JD, Weljie AM, Baur JA, Kasper DL, Arany Z. A branched-chain amino acid metabolite drives vascular fatty acid transport and causes insulin resistance. Nat Med. 2016; 22:421–426. [PubMed: 26950361]

Jong-Yeon K, Hickner RC, Dohm GL, Houmard JA. Long- and medium-chain fatty acid oxidation is increased in exercise-trained human skeletal muscle. Metabolism. 2002; 51:460–464. [PubMed: 11912554]

Katz A, Sharp RL, King DS, Costill DL, Fink WJ. Effect of high intensity interval training on 2,3-diphosphoglycerate at rest and after maximal exercise. Eur J Appl Physiol Occup Physiol. 1984; 52:331–335. [PubMed: 6539689]

Kelley DE. Skeletal muscle fat oxidation: timing and flexibility are everything. J Clin Invest. 2005; 115:1699–1702. [PubMed: 16007246]

Kelley DE, Goodpaster B, Wing RR, Simoneau JA. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. Am J Physiol. 1999; 277:E1130–1141. [PubMed: 10600804]

Kelley DE, He J, Menshikova EV, Ritov VB. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes. 2002; 51:2944–2950. [PubMed: 12351431]

Kelley DE, Simoneau JA. Impaired free fatty acid utilization by skeletal muscle in non-insulin-dependent diabetes mellitus. J Clin Invest. 1994; 94:2349–2356. [PubMed: 7989591]

Koeth RA, Levison BS, Culley MK, Buffa JA, Wang Z, Gregory JC, Org E, Wu Y, Li L, Smith JD, Tang WH, DiDonato JA, Lusis AJ, Hazen SL. gamma-Butyrobetaine is a proatherogenic intermediate in gut microbial metabolism of L-carnitine to TMAO. Cell Metab. 2014; 20:799–812. [PubMed: 25440057]

Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, Bain J, Stevens R, Dyck JR, Newgard CB, Lopaschuk GD, Muoio DM. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. Cell Metab. 2008; 7:45–56. [PubMed: 18177724]

Krssak M, Falk Petersen K, Dresner A, DiPietro L, Vogel SM, Rothman DL, Roden M, Shulman GI. Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. Diabetologia. 1999; 42:113–116. [PubMed: 10027589]

Kuhl JE, Ruderman NB, Musi N, Goodyear LJ, Patti ME, Crunkhorn S, Dronamraju D, Thorell A, Nygren J, Ljungkvist O, Degerblad M, Stahle A, Brismar TB, Andersen KL, Saha AK, Efendic S, Bavenholm PN. Exercise training decreases the concentration of malonyl-CoA and increases the expression and activity of malonyl-CoA decarboxylase in human muscle. Am J Physiol Endocrinol Metab. 2006; 290:E1296–1303. [PubMed: 16434556]

Lehmann R, Zhao X, Weigert C, Simon P, Fehrenbach E, Fritsche J, Machann J, Schick F, Wang J, Hoene M, Schleicher ED, Haring HU, Xu G, Niess AM. Medium chain acylcarnitines dominate the metabolite pattern in humans under moderate intensity exercise and support lipid oxidation. PLoS One. 2010; 5:e11519. [PubMed: 20634953]

Libert R, Van Hoof F, Laus G, De Nayer P, Habib Jiwan JL, de Hoffmann E, Schanck A. Identification of ethylsuccinylcarnitine present in some human urines. Clin Chim Acta. 2005; 355:145–151. [PubMed: 15820489]

Light AR, Hughen RW, Zhang J, Rainier J, Liu Z, Lee J. Dorsal root ganglion neurons innervating skeletal muscle respond to physiological combinations of protons, ATP, and lactate mediated by ASIC, P2X, and TRPV1. J Neurophysiol. 2008; 100:1184–1201. [PubMed: 18509077]

Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signalling and insulin resistance. Nat Rev Endocrinol. 2014; 10:723–736. [PubMed: 25287287]

Malenfant P, Joanisse DR, Theriault R, Goodpaster BH, Kelley DE, Simoneau JA. Fat content in individual muscle fibers of lean and obese subjects. Int J Obes Relat Metab Disord. 2001; 25:1316–1321. [PubMed: 11571593]

Mandarino LJ, Consoli A, Jain A, Kelley DE. Interaction of carbohydrate and fat fuels in human skeletal muscle: impact of obesity and NIDDM. Am J Physiol. 1996; 270:E463–470. [PubMed: 8638694]

McCoin CS, Knotts TA, Adams SH. Acylcarnitines–old actors auditioning for new roles in metabolic physiology. Nat Rev Endocrinol. 2015a; 11:617–625. [PubMed: 26303601]

McCoin CS, Knotts TA, Ono-Moore KD, Oort PJ, Adams SH. Long-chain acylcarnitines activate cell stress and myokine release in C2C12 myotubes: calcium-dependent and -independent effects. Am J Physiol Endocrinol Metab. 2015b; 308:E990–E1000. [PubMed: 25852008]

McGarry JD. Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. Diabetes. 2002; 51:7–18. [PubMed: 11756317]

Menshikova EV, Ritov VB, Ferrell RE, Azuma K, Goodpaster BH, Kelley DE. Characteristics of skeletal muscle mitochondrial biogenesis induced by moderate-intensity exercise and weight loss in obesity. J Appl Physiol (1985). 2007; 103:21–27. [PubMed: 17332268]

Menshikova EV, Ritov VB, Toledo FG, Ferrell RE, Goodpaster BH, Kelley DE. Effects of weight loss and physical activity on skeletal muscle mitochondrial function in obesity. Am J Physiol Endocrinol Metab. 2005; 288:E818–825. [PubMed: 15585590]

Mensink M, Blaak EE, van Baak MA, Wagenmakers AJ, Saris WH. Plasma free Fatty Acid uptake and oxidation are already diminished in subjects at high risk for developing type 2 diabetes. Diabetes. 2001; 50:2548–2554. [PubMed: 11679433]

Mihalik SJ, Goodpaster BH, Kelley DE, Chace DH, Vockley J, Toledo FG, DeLany JP. Increased levels of plasma acylcarnitines in obesity and type 2 diabetes and identification of a marker of glucolipotoxicity. Obesity (Silver Spring). 2010; 18:1695–1700. [PubMed: 20111019]

Mingrone G, Rosa G, Greco AV, Manco M, Vega N, Nanni G, Castagneto M, Vidal H. Intramyocitic lipid accumulation and SREBP-1c expression are related to insulin resistance and cardiovascular risk in morbid obesity. Atherosclerosis. 2003; 170:155–161. [PubMed: 12957694]

Minkler PE, Stoll MS, Ingalls ST, Kerner J, Hoppel CL. Quantitative acylcarnitine determination by UHPLC-MS/MS–Going beyond tandem MS acylcarnitine “profiles”. Mol Genet Metab. 2015a; 116:231–241. [PubMed: 26458767]

Minkler PE, Stoll MS, Ingalls ST, Kerner J, Hoppel CL. Validated method for the quantification of free and total carnitine, butyrobetaine, and acylcarnitines in biological samples. Anal Chem. 2015b; 87:8994–9001. [PubMed: 26270397]

Moder M, Kiessling A, Loster H, Bruggemann L. The pattern of urinary acylcarnitines determined by electrospray mass spectrometry: a new tool in the diagnosis of diabetes mellitus. Anal Bioanal Chem. 2003; 375:200–210. [PubMed: 12560963]

Mogensen M, Sahlin K, Fernstrom M, Glintborg D, Vind BF, Beck-Nielsen H, Hojlund K. Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes. 2007; 56:1592–1599. [PubMed: 17351150]

Moors CC, van der Zijl NJ, Diamant M, Blaak EE, Goossens GH. Impaired insulin sensitivity is accompanied by disturbances in skeletal muscle fatty acid handling in subjects with impaired glucose metabolism. Int J Obes (Lond). 2012; 36:709–717. [PubMed: 21712806]

Morino K, Petersen KF, Dufour S, Befroy D, Frattini J, Shatzkes N, Neschen S, White MF, Bilz S, Sono S, Pypaert M, Shulman GI. Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. J Clin Invest. 2005; 115:3587–3593. [PubMed: 16284649]

Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. N Engl J Med. 2004; 350:664–671. [PubMed: 14960743]

Petersen KF, Dufour S, Morino K, Yoo PS, Cline GW, Shulman GI. Reversal of muscle insulin resistance by weight reduction in young, lean, insulin-resistant offspring of parents with type 2 diabetes. Proc Natl Acad Sci U S A. 2012; 109:8236–8240. [PubMed: 22547801]

Piccolo BD, Comerford KB, Karakas SE, Knotts TA, Fiehn O, Adams SH. Whey protein supplementation does not alter plasma branched-chained amino acid profiles but results in unique metabolomics patterns in obese women enrolled in an 8-week weight loss trial. J Nutr. 2015; 145:691–700. [PubMed: 25833773]

Ritov VB, Menshikova EV, He J, Ferrell RE, Goodpaster BH, Kelley DE. Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. Diabetes. 2005; 54:8–14. [PubMed: 15616005]

Rodriguez-Gutierrez R, Lavalle-Gonzalez FJ, Martinez-Garza LE, Landeros-Olvera E, Lopez-Alvarenga JC, Torres-Sepulveda MR, Gonzalez-Gonzalez JG, Mancillas-Adame LG, Salazar-Gonzalez B, Villarreal-Perez JZ. Impact of an exercise program on acylcarnitines in obesity: a prospective controlled study. J Int Soc Sports Nutr. 2012; 9:22. [PubMed: 22574901]

Romijn JA, Coyle EF, Sidossis LS, Gastaldelli A, Horowitz JF, Endert E, Wolfe RR. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. Am J Physiol. 1993; 265:E380–391. [PubMed: 8214047]

Rutkowsky JM, Knotts TA, Ono-Moore KD, McCoin CS, Huang S, Schneider D, Singh S, Adams SH, Hwang DH. Acylcarnitines activate proinflammatory signaling pathways. Am J Physiol Endocrinol Metab. 2014; 306:E1378–1387. [PubMed: 24760988]

Sahlin K, Harris RC, Nylind B, Hultman E. Lactate content and pH in muscle obtained after dynamic exercise. Pflugers Arch. 1976; 367:143–149. [PubMed: 13343]

Schrauwen P, Hesselink MK. Reduced tricarboxylic acid cycle flux in type 2 diabetes mellitus? Diabetologia. 2008; 51:1694–1697. [PubMed: 18587560]

Short KR, Vittone JL, Bigelow ML, Proctor DN, Rizza RA, Coenen-Schimke JM, Nair KS. Impact of aerobic exercise training on age-related changes in insulin sensitivity and muscle oxidative capacity. Diabetes. 2003; 52:1888–1896. [PubMed: 12882902]

Ukropcova B, McNeil M, Sereda O, de Jonge L, Xie H, Bray GA, Smith SR. Dynamic changes in fat oxidation in human primary myocytes mirror metabolic characteristics of the donor. J Clin Invest. 2005; 115:1934–1941. [PubMed: 16007256]

van Hees AM, Jans A, Hul GB, Roche HM, Saris WH, Blaak EE. Skeletal muscle fatty acid handling in insulin resistant men. Obesity (Silver Spring). 2011; 19:1350–1359. [PubMed: 21331063]

Yang S, Minkler P, Hoppel C. cis-3,4-Methylene-heptanoylcarnitine: characterization and verification of the C8:1 acylcarnitine in human urine. J Chromatogr B Analyt Technol Biomed Life Sci. 2007; 857:251–258.

Zoppi CC, Silveira LR, Oliveira CA, Boschero AC, Curi R, Carneiro EM. Insulin release, peripheral insulin resistance and muscle function in protein malnutrition: a role of tricarboxylic acid cycle anaplerosis. Br J Nutr. 2010; 103:1237–1250. [PubMed: 19948081]

New Findings

What is the central question of this study?

Does improved metabolic health and insulin sensitivity following a weight loss and fitness intervention in sedentary, obese women alter exercise-associated fuel metabolism and incomplete mitochondrial fatty acid oxidation (FAO), as tracked by blood acylcarnitine patterns?

What is the main finding and its importance?

Despite improved fitness and blood sugar control, indices of incomplete mitochondrial FAO increased similarly in response to fixed load acute exercise bout; this indicates that intramitochondrial muscle FAO is inherently inefficient and is tethered directly to ATP turnover.

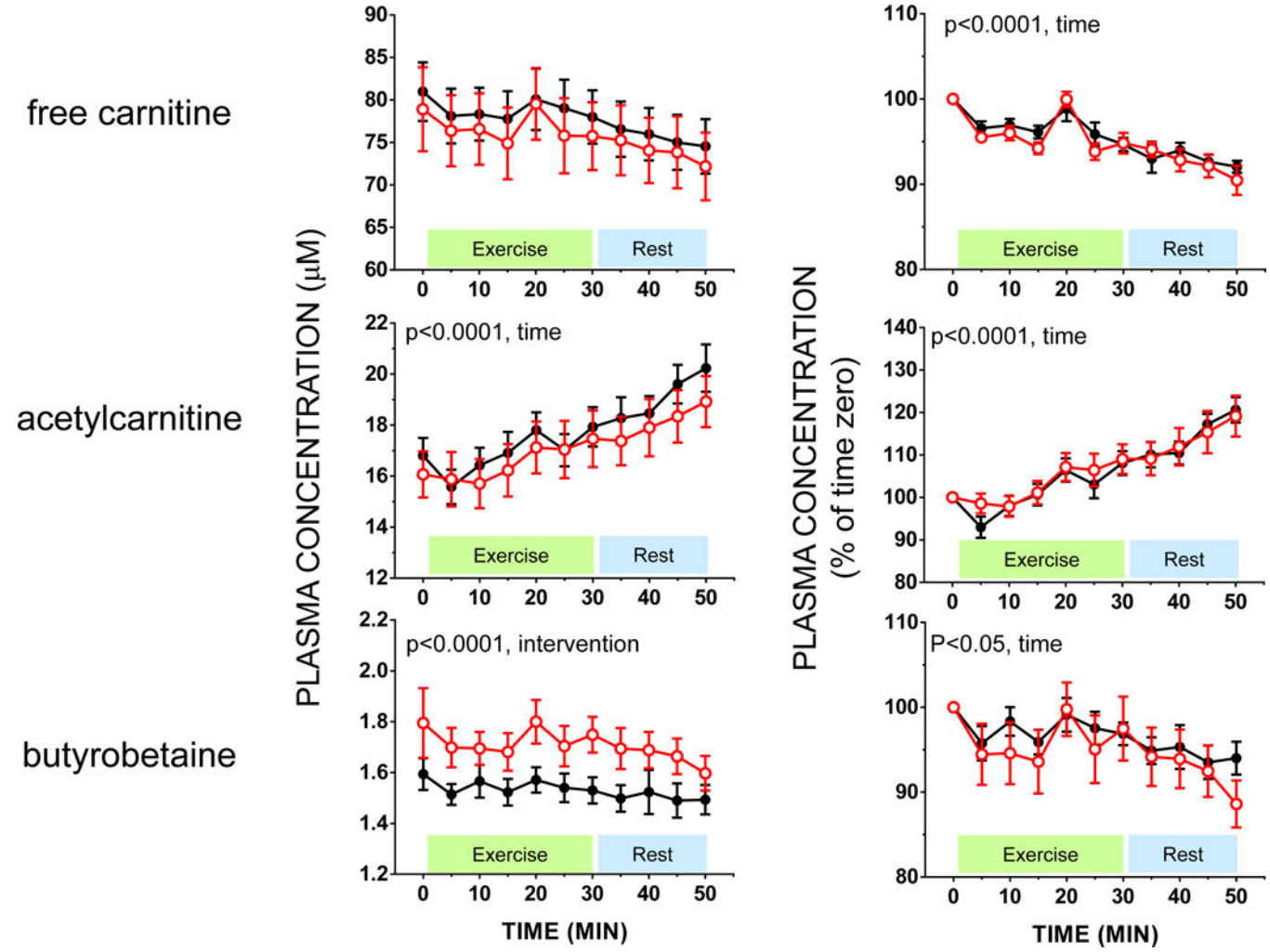

Figure 1. Patterns of plasma free carnitine, acetylcarnitine, and butyrobetaine in overnight-fasted women during an acute 30 minute exercise bout followed by 20 min rest, before (black, closed symbols) or after (red, open symbols) a weight loss and training intervention

Results are illustrated as concentration (left panels) or expressed as percentage of the zero minute, overnight-fasted value within each subject. Values are the mean ± SEM (n=15, pre-intervention; n=12 post-intervention); 2-way ANOVA (time, treatment, time × treatment interaction).

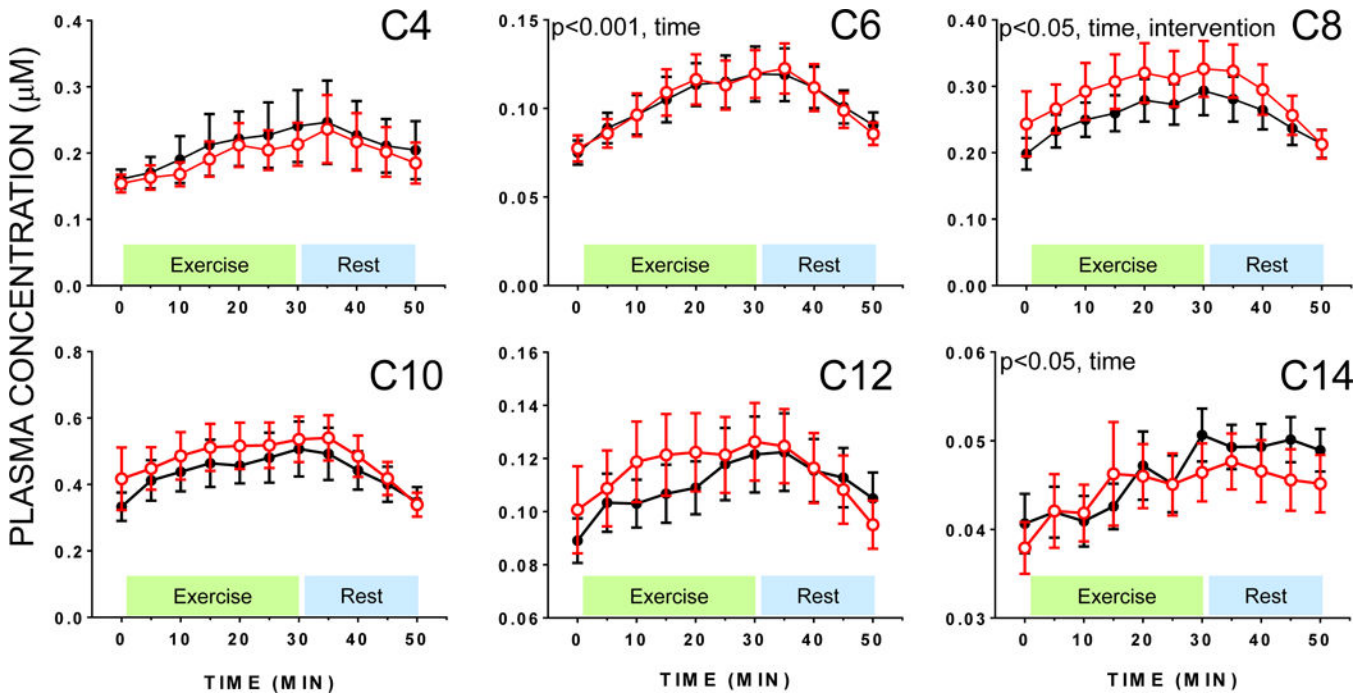

Figure 2. Patterns of plasma short- and medium-chain acylcarnitines in overnight-fasted women during an acute 30 minute exercise bout followed by 20 min rest, before (black, closed symbols) or after (red, open symbols) a weight loss and training intervention

Results are illustrated as concentration. Values are the mean ± SEM (n=15, pre-intervention; n=12 post-intervention); 2-way ANOVA (time, treatment, time × treatment interaction).

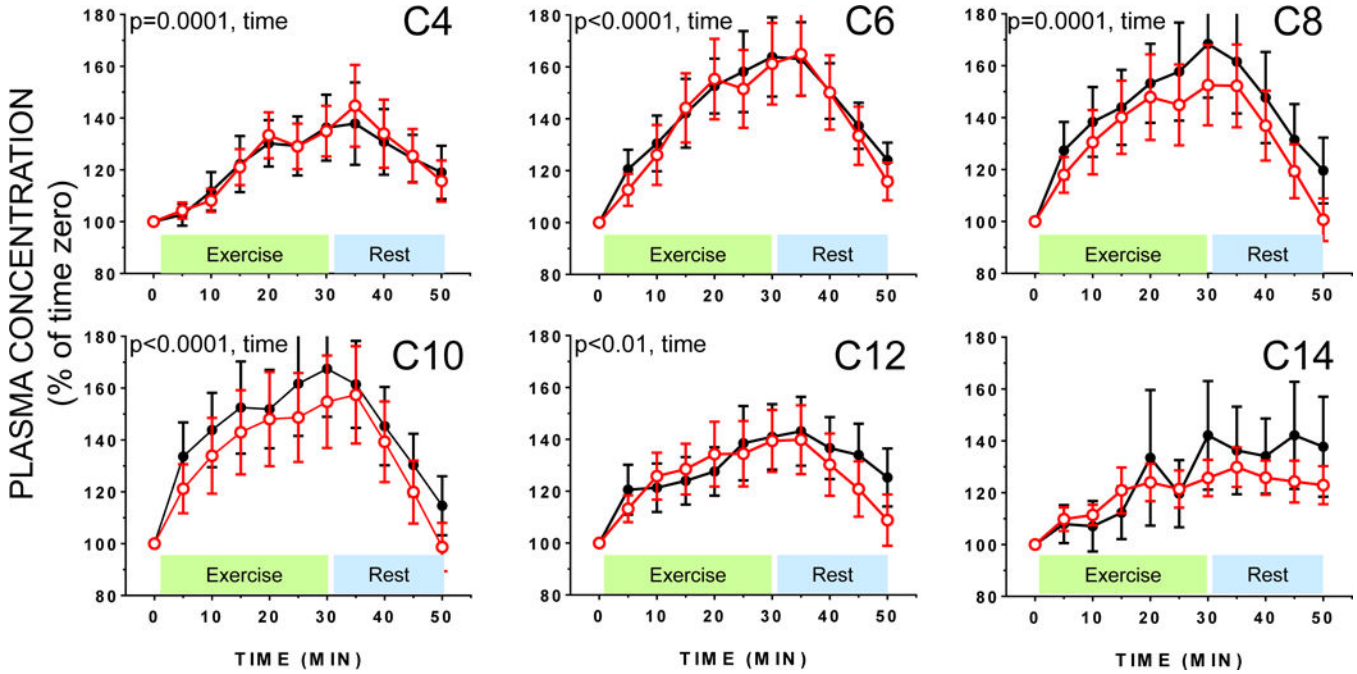

Figure 3. Patterns of plasma short- and medium-chain acylcarnitines in overnight-fasted women during an acute 30 minute exercise bout followed by 20 min rest, before (black, closed symbols) or after (red, open symbols) a weight loss and training intervention

Result expressed as percentage of the zero minute, overnight-fasted value within each subject. Values are the mean ± SEM (n=15, pre-intervention; n=12 post-intervention); 2-way ANOVA (time, treatment, time × treatment interaction).

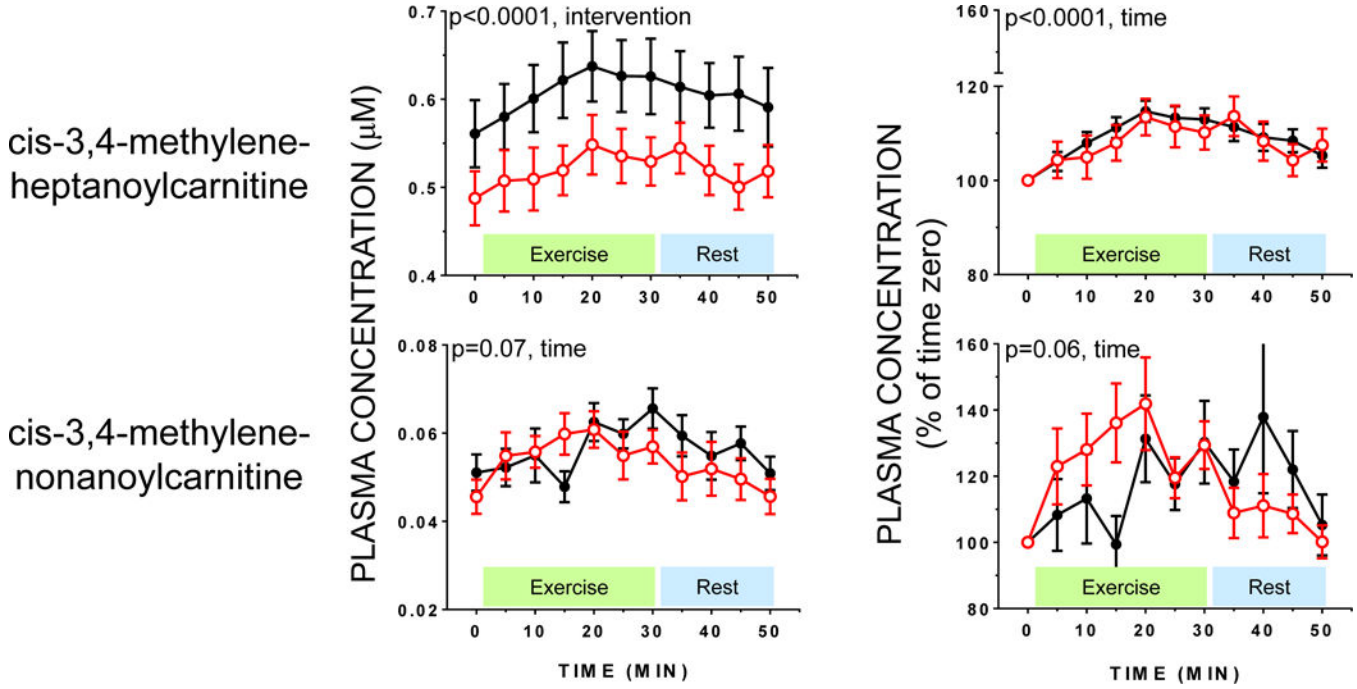

Figure 4. Patterns of plasma methylene-containing medium-chain acylcarnitines in overnight-fasted women during an acute 30 minute exercise bout followed by 20 min rest, before (black, closed symbols) or after (red, open symbols) a weight loss and training intervention

Results are illustrated as concentration (left panels) or expressed as percentage of the zero minute, overnight-fasted value within each subject. Values are the mean ± SEM (n=15, pre-intervention; n=12 post-intervention); 2-way ANOVA (time, treatment, time × treatment interaction).

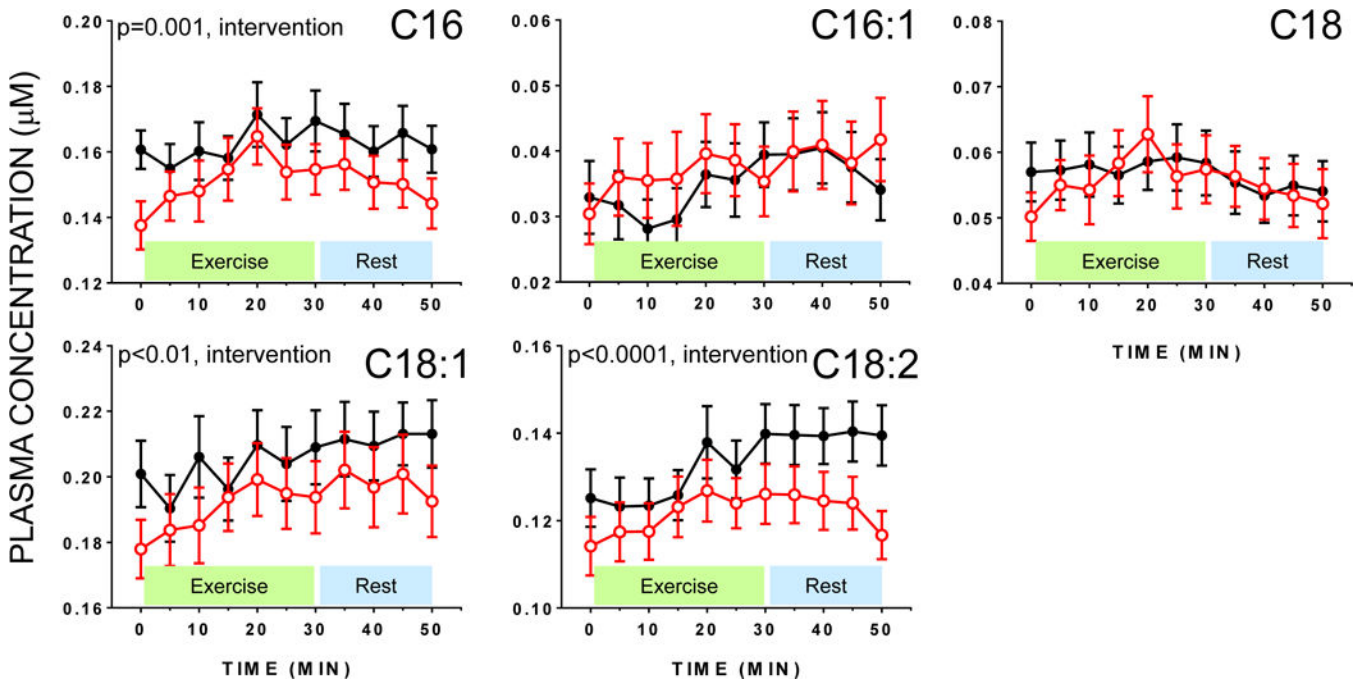

Figure 5. Patterns of plasma long-chain acylcarnitines in overnight-fasted women during an acute 30 minute exercise bout followed by 20 min rest, before (black, closed symbols) or after (red, open symbols) a weight loss and training intervention

Results are illustrated as concentration. Values are the mean ± SEM (n=15, pre-intervention; n=12 post-intervention); 2-way ANOVA (time, treatment, time × treatment interaction).

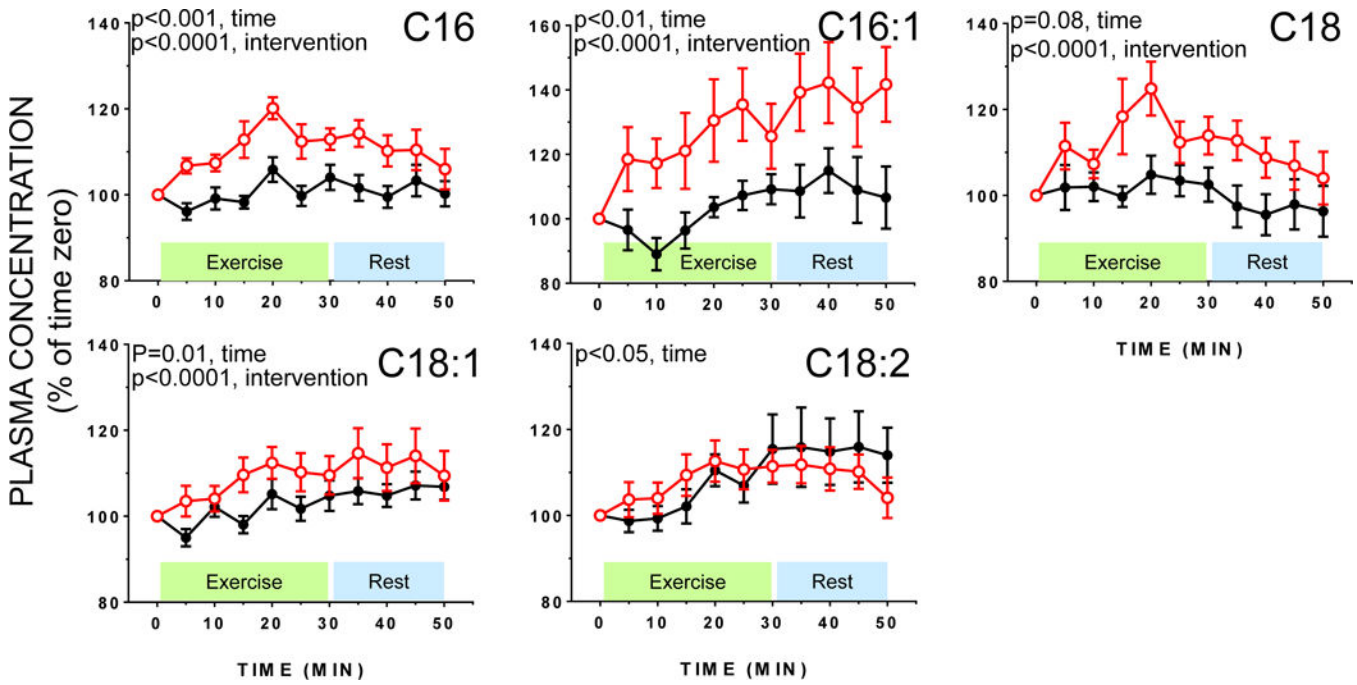

Figure 6. Patterns of plasma long-chain acylcarnitines in overnight-fasted women during an acute 30 minute exercise bout followed by 20 min rest, before (black, closed symbols) or after (red, open symbols) a weight loss and training intervention

Results are expressed as percentage of the zero minute, overnight-fasted value within each subject. Values are the mean ± SEM (n=15, pre-intervention; n=12 post-intervention); 2-way ANOVA (time, treatment, time × treatment interaction).

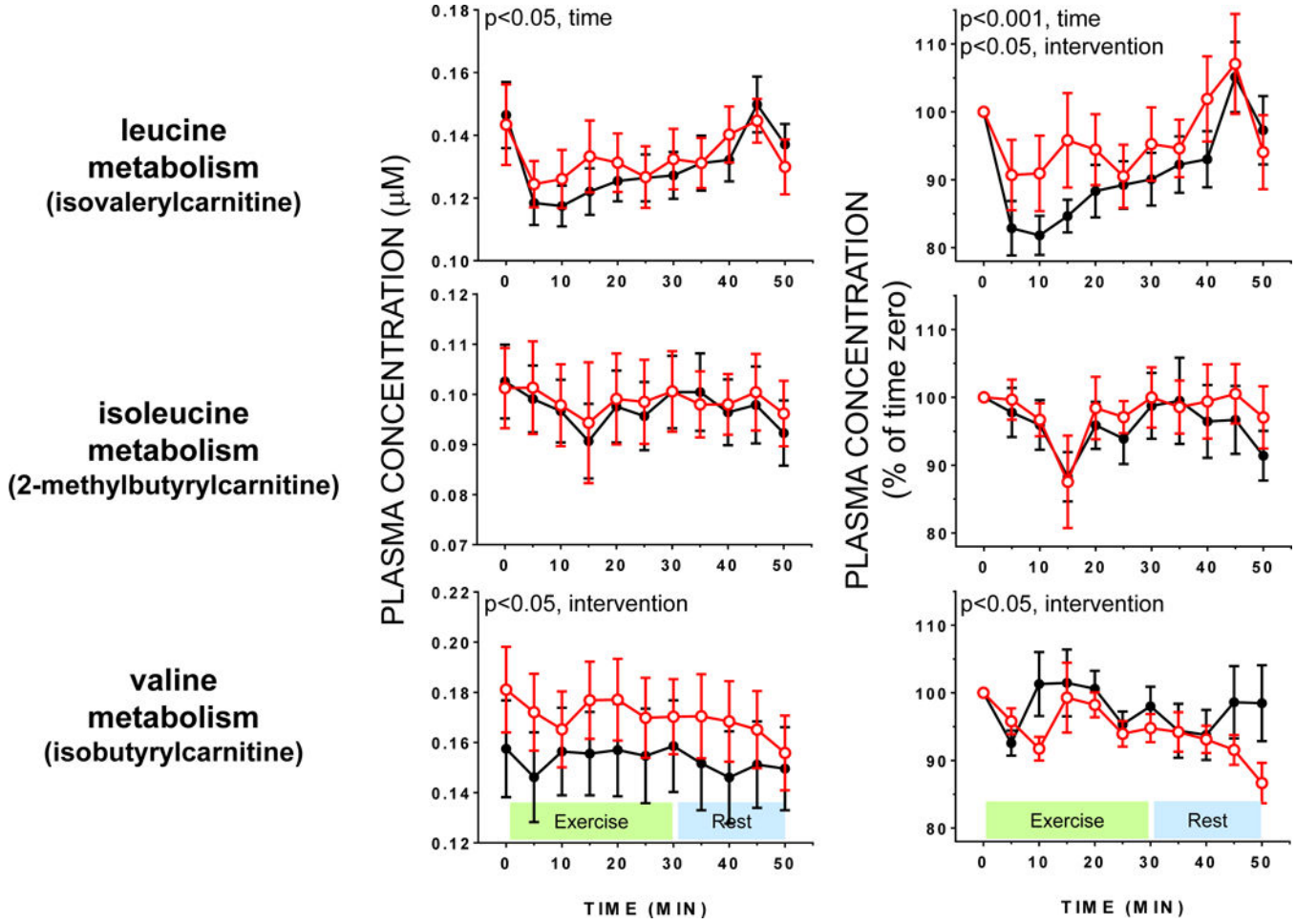

Figure 7. Patterns of plasma branched-chain amino acid derivatives in overnight-fasted women during an acute 30 minute exercise bout followed by 20 min rest, before (black, closed symbols) or after (red, open symbols) a weight loss and training intervention

Results are illustrated as concentration (left panels) or expressed as percentage of the zero minute, overnight-fasted value within each subject (right panels). Values are the mean ± SEM (n=15, pre-intervention; n=12 post-intervention); 2-way ANOVA (time, treatment, time × treatment interaction).

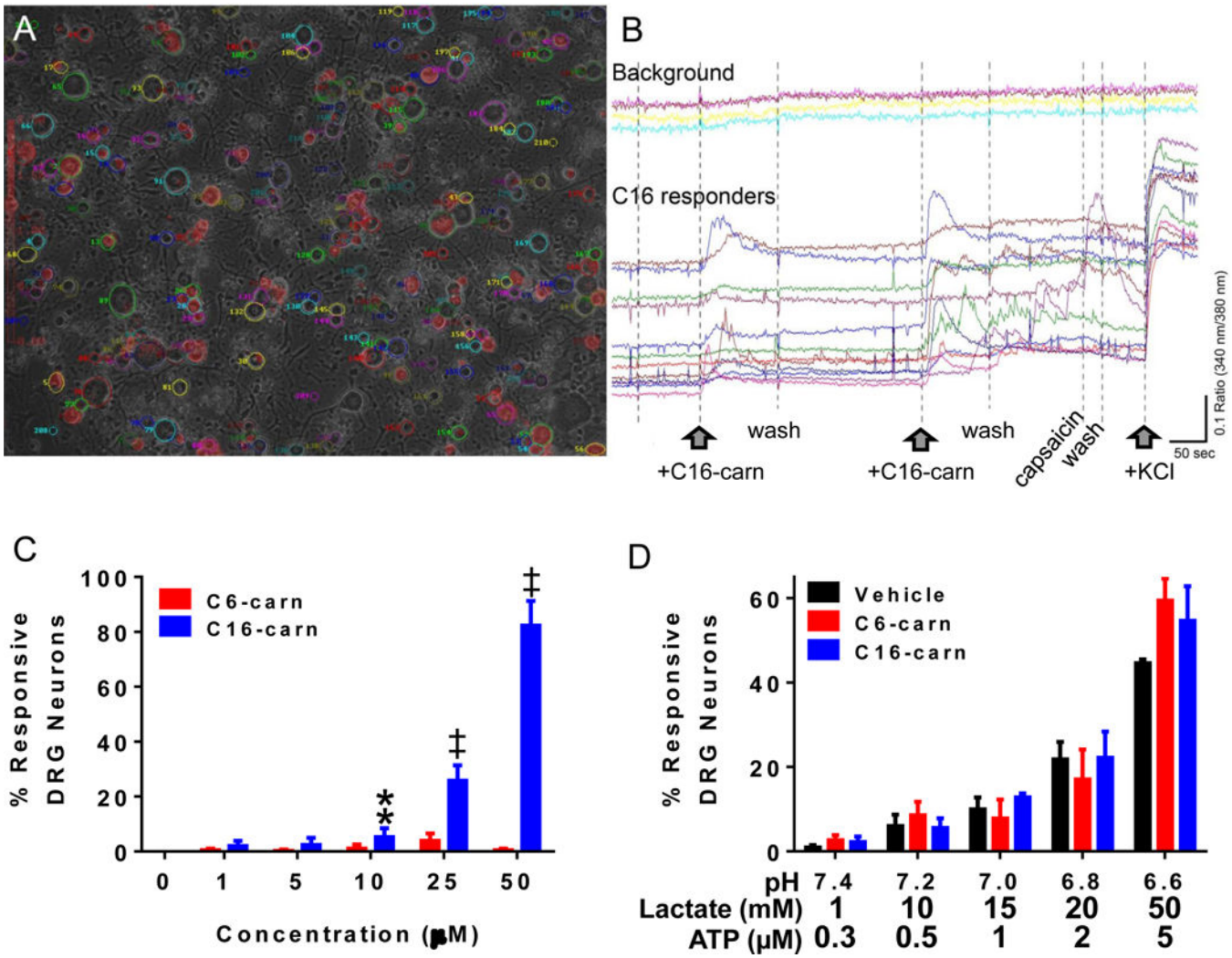

Figure 8. Responses of muscle-innervating dorsal root ganglion (DRG) neurons to palmitoyl- (C16) or hexanoylcarnitine (C6) treatment

(A) Image illustrating sub-populations of isolated DRG neurons positive for DiI dye (see Methods) in which changes in intracellular Ca^{2+} (Ca^{2+}_i) were analyzed. **(B)** Illustrative traces of Ca^{2+}_i responses in individual DiI-positive neurons and non-cell background control regions in response to vehicle or transient 25 μM palmitoylcarnitine (C16-carn). Depicted are changes in response to C16-carn, washout (Wash), capsaicin (Cap) or KCl. **(C)** Percentage of total DiI-positive DRG neurons responsive to hexanoylcarnitine (C6-carn; red bars) or palmitoylcarnitine (C16-carn; blue bars); no vehicle-treated cells responded above the threshold of detection. **(D)** Percentage of total DiI-positive DRG neurons responsive to C6-carn (red bars) or C16-carn (blue bars) in the presence of lactate, ATP and protons. There was a significant effect of pH/lactate/ATP treatments on neuron activation ($p<0.0001$; 2-way ANOVA), but no significant interaction with acylcarnitines. Sample sizes varied by condition and treatment. Panel C: C6-carn studies, n=20, 3, 5, 3, 4, 5 (vehicle, 1, 5, 10, 25, 50 μM, respectively); C16-carn studies, n=27, 4, 5, 8, 6, 4 (vehicle, 1, 5, 10, 25, 50 μM, respectively). Panel D, at pH 7.4, 7.2, 7.0, 6.8, 6.6, respectively: n=10, 6, 15, 11, 3 (vehicle);

n=23, 4, 5, 6, 8 (C6-carn); n=18, 4, 4, 5, 6 (C16-carn). **p<0.01; ‡p<0.001 compared to vehicle, Kruskal-Wallis test

Table 1

Body mass, fitness, and fuel homeostasis indices in previously sedentary obese women following a weight loss and fitness intervention.

	Pre-intervention[a]	**Post-intervention**[b]	**p-value**[c]
Body Mass (kg)	88.6 ± 2.1	83.0 ± 3.1	<0.0001
BMI, kg/m^2	33.5 ± 0.6	31.0 ± 0.9	<0.0001
Body Fat%	47.5 ± 1.0	43.4 ± 1.5	0.0002
Fat Mass (kg)	41.9 ± 1.6	36.2 ± 2.4	<0.0001
Fat Free Mass (FFM, kg)	43.1 ± 0.8	43.3 ± 0.9	NS
VO_2peak (mL/kg/min)	21.1 ± 1.1	25.6 ± 1.1	<0.0001
VO_2peak (mL/kg FFM/min)	43.7 ± 1.6	49.4 ± 1.4	<0.0001
Maximal Power (Watts at VO_2peak)	142.0 ± 3.8	166.7 ± 6.4	<0.0001
Exercise bout heart rate (bpm)	108.8 ± 1.8	101.0 ± 2.6	0.0003
Exercise bout energy from CHO (kcal)	79.4 ± 7.2	65.6 ± 4.0	0.0413
Exercise bout energy from fat (kcal)	46.4 ± 7.1	53.0 ± 6.7	NS
Exercise bout RER	0.8775 ± 0.0142	0.8575 ± 0.0116	NS
Resting energy expenditure (kcal/kg/d)	17.8 ± 0.5	19.4 ± 0.7	0.0032
Resting energy expenditure (kcal/kg FFM/d)	36.5 ± 0.7	36.9 ± 1.2	NS
Resting RER	0.8308 ± 0.0076	0.8117 ± 0.0098	0.0765

[a] pre-diet and exercise intervention, n=15

[b] post-diet and exercise intervention, n=12

[c] comparisons using paired t-tests from n=12 subjects completing both Test Week 1 (pre-intervention) and Test Week 2 (post-intervention)

Values are means ± SEM; NS = not statistically significant

Some variables have been presented previously (Campbell *et al.*, 2014)

Table 2

Plasma total carnitine, free carnitine, and acylcarnitine concentrations (μM) in overnight-fasted sedentary, insulin-resistant obese women before and after a weight loss and exercise training intervention that improved fitness and insulin sensitivity

	Pre-intervention[a]	Post-intervention[b]
total carnitine	102.12 ± 3.41	99.62 ± 5.18
free carnitine	80.98 ± 3.45	78.91 ± 4.95
γ-butyrobetaine	1.59 ± 0.06	1.80 ± 0.14
acetylcarnitine	16.81 ± 0.69	16.07 ± 0.91
propionylcarnitine	0.70 ± 0.04	0.69 ± 0.08
butyrylcarnitine	0.16 ± 0.02	0.15 ± 0.01
isobutyrylcarnitine	0.16 ± 0.02	0.18 ± 0.02
R-3-hydroxy-butyrylcarnitine	0.07 ± 0.008	0.06 ± 0.006
S-3-hydroxy-butyrylcarnitine	0.08 ± 0.006	0.07 ± 0.008
valerylcarnitine	0.01 ± 0.002	0.02 ± 0.002
isovalerylcarnitine	0.15 ± 0.01	0.14 ± 0.01
3-hydroxy-isovalerylcarnitine	0.06 ± 0.007	0.06 ± 0.008
2-methyl-butyrylcarnitine	0.10 ± 0.007	0.101 ± 0.008
pivaloylcarnitine	0.02 ± 0.003	0.01 ± 0.003
tigloylcarnitine	0.02 ± 0.002	0.02 ± 0.002
3-methyl-crotonylcarnitine	<0.01	<0.01
hexanoylcarnitine	0.08 ± 0.007	0.08 ± 0.007
R-3-hydroxy-hexanoylcarnitine	0.02 ± 0.002	0.01 ± 0.003
S-3-hydroxy-hexanoylcarnitine	0.03 ± 0.003	0.025 ± 0.004
phenylacetylcarnitine	0.05 ± 0.006	0.05 ± 0.01
phenylpropionylcarnitine	0.01 ± 0.002	0.01 ± 0.002
4-phenyl-butyrylcarnitine	<0.01	<0.01
benzoylcarnitine	0.02 ± 0.002	0.02 ± 0.36
4-methyl-hexanoylcarnitine	0.02 ± 0.002	0.02 ± 0.002
octanoylcarnitine	0.20 ± 0.02	0.24 ± 0.05
R-3-hydroxy-octanoylcarnitine	0.06 ± 0.004	0.06 ± 0.005
S-3-hydroxy-octanoylcarnitine	0.16 ± 0.01	0.18 ± 0.02
valproylcarnitine	0.02 ± 0.002	0.02 ± 0.002
cis-3,4-methylene-heptanoylcarnitine	0.56 ± 0.04	0.49 ± 0.03
4-methyl-octanoylcarnitine	0.02 ± 0.002	0.02 ± 0.002
2,6-dimethyl-heptanoylcarnitine	0.02 ± 0.003	0.02 ± 0.002
decanoylcarnitine	0.33 ± 0.04	0.42 ± 0.09
cis-4-decenoylcarnitine	0.18 ± 0.02	0.19 ± 0.03
cis-3,4-methylene-nonanoylcarnitine	0.05 ± 0.004	0.05 ± 0.004
R-3-hydroxy-decanoylcarnitine	0.05 ± 0.005	0.05 ± 0.005

	Pre-intervention[a]	**Post-intervention**[b]
S-3-hydroxy-decanoylcarnitine	0.12 ± 0.01	0.13 ± 0.02
5-decynoylcarnitine	0.01 ± 0.002	0.01 ± 0.002
lauroylcarnitine	0.09 ± 0.008	0.10 ± 0.02
trans-2-dodecenoylcarnitine	0.04 ± 0.003	0.03 ± 0.003
R-3-hydroxy-lauroylcarnitine	0.01 ± 0.002	<0.01
S-3-hydroxy-lauroylcarnitine	0.02 ± 0.003	0.01 ± 0.003
myristoylcarnitine	0.04 ± 0.003	0.04 ± 0.003
myristoleoylcarnitine	0.03 ± 0.003	0.03 ± 0.004
cis-5-tetradecenoylcarnitine	0.12 ± 0.01	0.12 ± 0.02
trans-2-tetradecenoylcarnitine	0.03 ± 0.003	0.02 ± 0.003
cis,cis-5,8-tetradecadienoylcarnitine	0.09 ± 0.009	0.10 ± 0.01
R-3-hydroxy-myristoylcarnitine	0.03 ± 0.002	0.02 ± 0.003
S-3-hydroxy-myristoylcarnitine	<0.01	<0.01
palmitoylcarnitine	**0.16 ± 0.006**	**0.14 ± 0.007** *
palmitoleoylcarnitine	0.03 ± 0.006	0.03 ± 0.005
trans-2-hexadecenoylcarnitine	0.01 ± 0.002	0.01 ± 0.002
R-3-hydroxy-palmitoylcarnitine	0.02 ± 0.002	0.02 ± 0.002
S-3-hydroxy-palmitoylcarnitine	0.01 ± 0.003	0.01 ± 0.003
stearoylcarnitine	0.06 ± 0.004	0.05 ± 0.004
oleoylcarnitine	**0.20 ± 0.01**	**0.18 ± 0.009** *
linoleoylcarnitine	0.12 ± 0.007	0.11 ± 0.007
alpha-linolenoylcarnitine	0.02 ± 0.002	0.02 ± 0.002
gamma-linolenoylcarnitine	0.02 ± 0.002	0.02 ± 0.002
R-3-hydroxy-stearoylcarnitine	0.02 ± 0.002	0.02 ± 0.002
S-3-hydroxy-stearoylcarnitine	0.01 ± 0.003	<0.01
malonylcarnitine	0.03 ± 0.004	0.03 ± 0.003
succinylcarnitine	0.06 ± 0.005	0.07 ± 0.008
methyl-malonylcarnitine	0.04 ± 0.003	0.05 ± 0.004
ethyl-malonylcarnitine	<0.01	<0.01
glutaroylcarnitine	0.09 ± 0.007	0.10 ± 0.01
adipoylcarnitine	0.02 ± 0.004	0.02 ± 0.003
3-methyl-glutaroylcarnitine	0.06 ± 0.006	0.05 ± 0.007
suberoylcarnitine	0.03 ± 0.003	0.02 ± 0.003
sebacoylcarnitine	0.02 ± 0.002	0.02 ± 0.002

[a] pre-diet and exercise intervention, n=15

[b] post-diet and exercise intervention, n=12

* p<0.05, comparisons using paired t-tests from n=12 subjects completing both Test Week 1 (pre-intervention) and Test Week 2 (post-intervention)

Values are means ± SEM; NS = not statistically significant